STUDIES IN THE BIOSYNTHESIS OF THE HORMONES OF THE THYROID GLAND.

By

Brian Denis Wilson. B.Sc.(Hons.)

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CONTENTS.

INTRODUCTION

This project was undertaken in order to elucidate two important problems in Thyroid Biochemistry namely, the kinetics of labelling of tyrosine and monoiodotyrosine (MIT) and the pathways of synthesis of triiodothyronine (T_3) and thyroxine (T_4).

Several hypotheses for the formation of T_3 and T_4 have been postulated:

A Pathways of synthesis of tri-iodothyronine.

1) Coupling of monoiodotyrosine and di-iodotyrosine (DIT).

2) Deiodination of T_4.

B Pathways of synthesis of thyroxine.

1) Coupling of two molecules of DIT.

2) Iodination of T_3.

There is evidence supporting all these hypotheses. Therefore, at present the pathway of T_3 and T_4 biosynthesis remains undecided.

The main line of approach adopted in this study was (a) to measure the relative distribution of the ^{131}I between the thyroidal iodoamino acids and

(b) to determine the variation in the absolute specific activity of the iodine in the different iodoamino acids as a function of time. These parameters were measured in rats on adequate and low-iodine diets and in rats treated with propylthiouracil (PTU).

Evidence will be presented supporting the following hypotheses:

1) that T_4 is synthesized from the coupling of two molecules of DIT.
2) that T_3 is synthesized from the coupling of one molecule of MIT and one molecule of DIT.

Although no simple precursor-product relationship was found between MIT and DIT, evidence will also be presented showing that MIT is the precursor of DIT.

A discussion of the relavent literature will be found in the introductions to Chapters 2 - 5 and in the discussions at the end of Chapters 2 - 5.

CHAPTER 1.

MATERIALS AND METHODS.

Experimental Animals.

Male Sprague-Dawley or Wistar rats weighing 270 - 330g. were used. The animals were selected for the individual experiments so that the variation in weight was not greater than ± 15 g.

The stock from which the experimental animals originated was fed, for many years, a commercial pellet diet (manufactured by Vereeniging Milling Co.). Stable iodine analysis by the alkaline ashing procedure (Chapter 2.) on five batches of the diet gave 30.2 ± 3.3 µg. iodine/100g. (mean ± S.D.). Rats selected for each experiment were maintained, from weaning time until commencement of the experiment, on the commercial pellet diet and had free access to tap water. The iodine content of the tap water was 0.3 µg. iodine/100 ml. All experimental animals were housed for at least two months before the commencement of the experiments and also throughout the experiments, in a constant temperature room at 21 - 23°.

Administration of ^{131}I iodide.

Radioiodine was obtained as carrier-free $Na^{131}I$ from the Radiochemical Centre, Amersham, Bucks. After dilution with 0.9% NaCl, 0.3 ml. of the ^{131}I solution was injected into rats either intraperitoneally for experiments of long duration or, intravenously under light ether anaesthesia for experiments of short duration.

The activity of ^{131}I injected per rat varied according to the type of experiment undertaken. Rats fed the pellet and low iodine diets received 50 - 200 µc. (depending on the duration of the experiment) whereas propylthiouracil-treated rats received as much as 500 µc. each.

Measurement of ^{131}I uptake by the thyroid gland.

The rats were killed by gassing with coal gas. After death the thyroid glands were immediately dissected, weighed, placed in all-glass semi-micro homogenizers and homogenized in 80 µl. 0.1M-thiouracil. The thiouracil was added in order to decrease any deiodination of the iodoamino acids and to prevent oxidation of inorganic iodide during homogenation and subsequent enzymatic digestion (Taurog, Potter and Chaikoff, 1955; Tong and Chaikoff, 1958; Taurog 1963a, 1963b, Inoue 1966.). For experiments of short (0 - 5 min.) duration, the thyroid

glands were immediately dissected and homogenized in thiouracil, and then boiled for 3 min.

After homogenation or boiling, the percentage uptake of ^{131}I by the thyroid glands was measured using either an open directional scintillation counter calibrated to 100 µc. at a distance of 50 cm. or, a ring counter (incorporating four G.M. tubes) similar to that described by Campbell, Cuthbertson, Matthews and McFarlane (1956). The ring counter was calibrated using the same ^{131}I standards employed for the directional scintillation counter.

Hydrolysis of the thyroid glands.

Immediately after the measurements of the percentage ^{131}I uptake, the individual thyroid homogenates were enzymically hydrolysed in the semi-micro homogenizers by a method similar to that described by Tong and Chaikoff (1958).

To each homogenate was added 0.2 ml. of a 1% solution of pancreatin and trypsin made up in 0.2 M-tris-HCl buffer, pH 8.4, 10 µl. 0.1 M-$MmCl_2$ and two drops of toluene. Using the pestle of each homogenizer, the contents of the homogenizers were stirred and then incubated at 40° for 24 hr. with occasional stirring. During incubation the volume of the hydrolysate decreased

to approximately 0.2 ml.

Paper chromatography and radioautography.

Chromatography papers 25 x 36 cm. were cut from large commercial sheets of Whatman No. 1 paper. The thyroid hydrolysates were applied as spots along 6 cm. strips marked on the chromatography paper. Approximately one-fifth of each hydrolysate was chromatographed in butan-1-ol equilibrated with 2N-acetic acid (BAW). The rest of each hydrolysate was chromatographed in butan-1-ol; dioxan: aq. 2N-NH_3 (BDA, 4 : 1 : 5 v/v/v). However, when the distribution of ^{127}I in the glands was determined, the hydrolysate was made up to 0.3 ml. and 50 µl. of the hydrolysate was chromatographed in BAW and 150 µl. in BDA. Chromatographically pure standards of iodide, MIT, DIT, T_3 and T_4 were chromatographed separately on the same papers as the thyroid hydrolysates.

The chromatographs were developed for 16-18 hr. at room temperature by the ascending technique. The resolution of MIT and DIT was accomplished in BAW while BDA resolved T_4 and T_3. Unless otherwise stated, the inorganic iodide fractions resolved in BAW were used for the measurement of specific activity or percentage distribution of thyroidal inorganic iodide.

After drying the chromatograms at room temperature, each chromatogram was wrapped in cellophane paper

and the ^{131}I compounds were located by radioautography (Ilford Ilfex non-screen X-ray film).

In order to determine the exposure time for radioautography, the position of highest activity on each chromatogram was located and counted, using a lead shielded mica end-window β -particle counter with either a 1 cm. or 0.2 cm. slit. The 0.2 cm. slit was used for the counting of BDA chromatograms whereas the 1 cm. slit was used for the counting of BAW chromatograms. The exposure time was assessed by applying the formula:

$$\text{Exposure time (hr.)} = \frac{100{,}000}{\text{maxium count/100 sec.}}$$

Under these conditions, a linear relationship existed between density on the X-ray film and radioactivity on the paper chromatogram. (See below under Assay of radioactivity).

After radioautography the sections of the chromatograms embodying the marker substances were cut off, sprayed first with diazotized sulphanilic acid and then with 5% $Na_2 CO_3$. Under these conditions the iodotyrosines and iodothyronines produce characteristic colours and can thus be located. The diazotized sulphanilic acid was prepared by adding 10 ml. cold 0.05M-sulphanilic acid in 9% HCl to 10 ml. 5% $NaNO_2$ and allowing the mixture to diazotize for 5 min. (Taurog,

Tong and Chaikoff, 1950).

The inorganic iodide was located by spraying with either 1% $PdCl_2$ (Gross and Leblond, 1951) or diazotized sulphanilic acid (van Zyl and Bhaga, 1961). In the latter method the colour fades within 2 min. so that the iodide area should be marked immediately. However, it was very convenient to use this method since both inorganic iodide and the iodoamino acids could be simultaneously identified.

The darkened bands on the X-ray film were identified according to the positions of the stable marker substances. On each chromatogram the sections corresponding to the darkened bands observed on the radioautograph were marked off and cut out. All possible precautions against contamination were taken. By keeping the experimental conditions the same, the R_F values of the iodocompounds remained constant so that it subsequently became possible to identify the bands on the X-ray film without use of stable marker substances.

The sections cut out from the chromatograms were assayed for their stable iodine content by a modified alkaline ashing method described in Chapter 2. and for their ^{131}I content (see below under Assay of radioactivity).

Completeness of chromatographic separations.

According to Plaskett, Barnaby and Lloyd (1963a), the chromatographic resolution of T_3 and T_4 is incomplete in BDA although radioautographs or the coloured regions on the chromatograms may indicate complete separations. The bands may appear to be completely resolved simply because the eye is too insensitive to detect any overlap between the bands.

For this reason it was desirable to study the resolution of T_3 and T_4 in BDA and, in addition, the resolution of MIT and DIT in BAW. The completeness of the chromatographic separations in BDA and BAW was determined by cutting out the sections of several chromatograms corresponding to the darkened areas of MIT, DIT, T_3 and T_4 observed on the radioautograph and counting in a well scintillation counter. The interpeak areas were also cut out and counted. The level of radioactivity between the MIT and DIT peaks was respectively 3.4% and 2.9% of the activity of the lower iodotyrosine peak for animals killed 2 hr. and 24 hr. after administration of ^{131}I. For the same time intervals the level of activity of the interpeak region of T_3 and T_4 was respectively 15.2% and 12.8% of the activity of the T_3 peak. Typical results of detailed scans of the iodotyrosine and iodothyronine

regions are illustrated in Fig. 1. and Fig. 2 . The chromatographic separations in BDA and BAW were considered adequate for the present work.

Particularly at the early intervals after injection of ^{131}I, when total stable iodine analyses of the various thyroidal iodoamino acids were not performed, rather less than the entire extent of each band required for specific activity analysis was cut out in order to minimise contamination from any neighbouring band.

Electrophoresis.

For the analysis of inorganic iodide in thyroid homogenates and thyroid hydrolysates, iodine analyses were performed on iodide separated from organic iodide by electrophoresis. When the quantity of inorganic iodide in thyroid homogenates was determined, the glands were rapidly dissected, weighed and then homogenized in 80 µl. 0.1 M-thiouracil in ice.

25 µl. of each of the test solutions was applied to cellulose acetate strips and subject to electrophoresis in veronal buffer pH 8.6, at a constant voltage of 280 V. for 25 min. Thereafter the strips were dried at room temperature, exposed to X-ray film and the sections of the electrophoresis strips corresponding to the darkened areas on the radioautographs were cut out and counted.

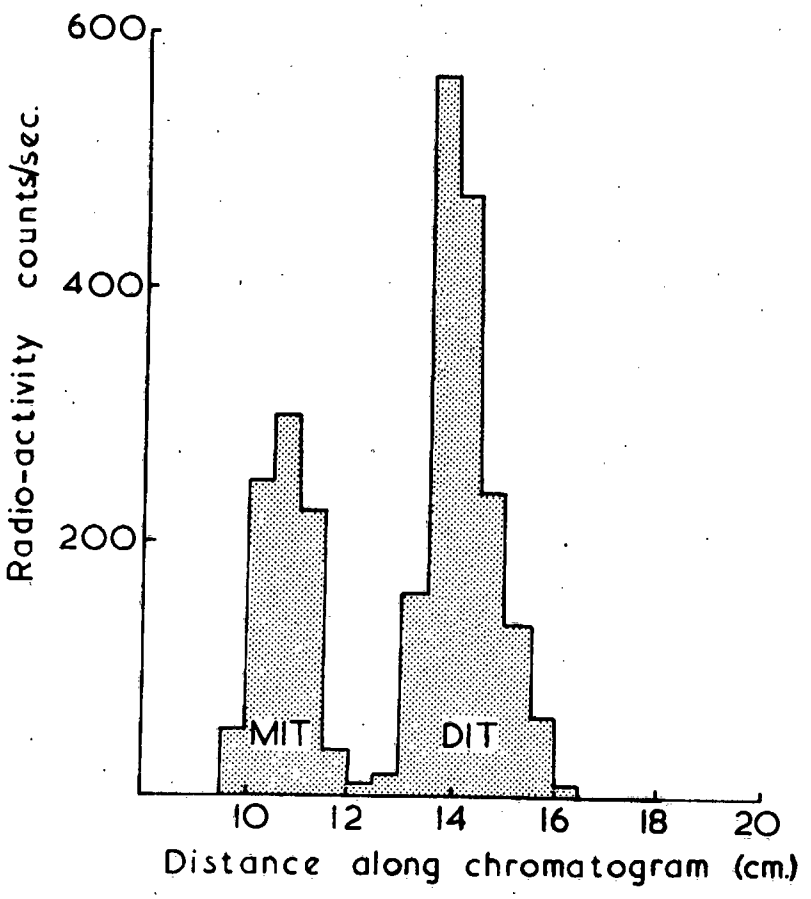

Fig. 1. Distribution of radioactivity on a chromatogram of a hydrolysate of rat thyroid 48 hr. after injection of ^{131}I. Detailed scanning of the iodotyrosine region.

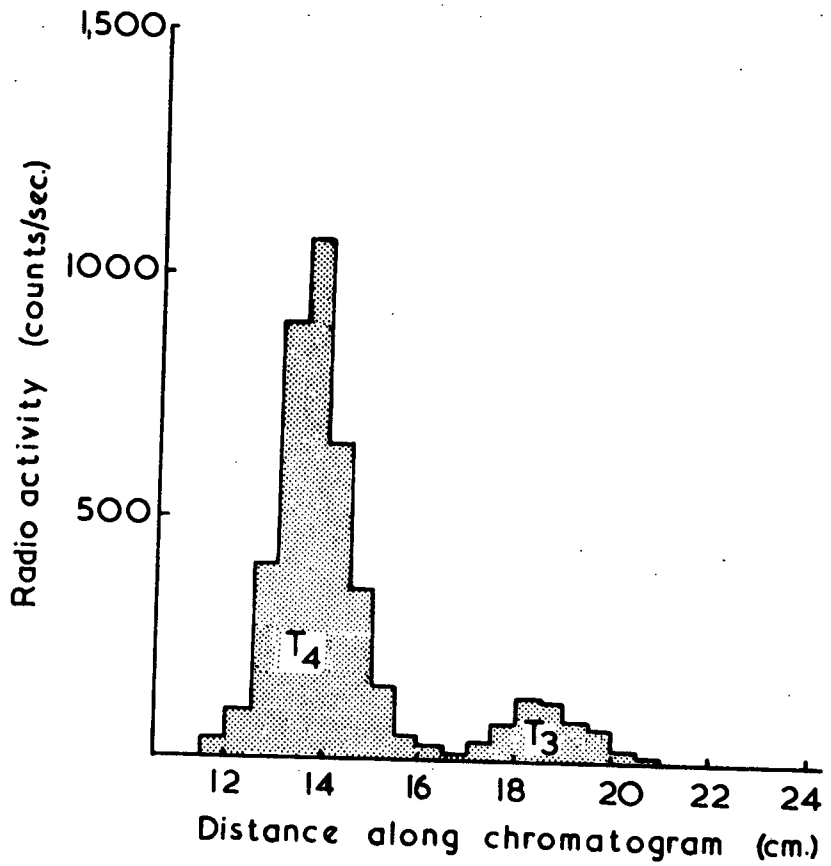

Fig. 2. Distribution of radioactivity on a chromatogram of a hydrolysate of rat thyroid 48 hr. after injection of ^{131}I. Detailed scanning of the iodothyronine region.

The inorganic iodide fractions were then extracted with 3 ml. KOH (3.57 ml.4N-KOH made up to 50 ml. with water) for 15 min. Portions of the extracts (0.5 ml.) were assayed for stable iodine by the colorimetric method described in Chapter 2 .

Assay of radioactivity.

Each 0.5 ml. sample of the radioactive ash solutions to be measured for estimation of stable iodine (see Chapter 2 , Colorimetric analysis of iodine) was first counted in a well-type scintillation counter (Ecko, 2 cm. crystal) in order to assay the ^{131}I content. Sufficient counts were taken for a statistical accuracy of at least 1%, except for the T_3 and iodide samples where a statistical accuracy of 2% or better was attained. The scintillation counter had been calibrated previously for ^{131}I up to 0.5 µc. for 0.5 ml. and 1.0 ml. samples. For specific activity measurements, all activities were corrected to the time of injection of ^{131}I.

The percentage distribution of the radioactivity between the various thyroidal iodoamino acids was assessed by one of the following three methods.

1) Cutting the chromatograms into strips 4 cm. wide and sectioning these strips into 0.5 cm. segments. Each segment was counted in a well-type scintillation counter.

2) Direct scanning with a shielded mica end-window β - particle counter connected to a rate meter and automatic recorder and determining the areas under the curves by planimetry.

3) Densitometric scanning of radioautographs using a Photovolt, transmission densitometer coupled with a multiplier photometer and Beckman 10 inch potentiometric recorder with disc integrator. A slit width of 0.1 cm. and an orange filter were used.

In most cases it was not possible to determine the percentage distribution of ^{131}I along the chromatograms by direct scanning before specific activity analysis, because of the large numbers of chromatograms to be scanned in each of the experiments. Therefore, since radioautographs had to be prepared, it was desirable to study the possibility of obtaining a quantitative estimate of the percentage distribution of ^{131}I along the chromatograms by densitometric scanning of the radioautographs. The distribution of the ^{131}I-labelled compounds could thus be assessed at a more convenient time subsequent to specific activity analysis.

A comparison was made between densitometric scanning of the radioautographs and direct counting of the chromatograms in assessing the relative proportions of the labelled thyroidal iodoamino acids. TABLE 1.

TABLE 1.

Measurement of the distribution of ^{131}I in rat thyroid hydrolysates* by direct counting of chromatograms and by densitometric scanning of radioautographs.

Time after ^{131}I	^{131}I component	direct counting** %	densitometric scanning ** %
4 hr.	origin	5.9 ± 0.1***	6.1 ± 0.3
	MIT	31.2 ± 0.9	31.1 ± 1.7
	DIT	47.5 ± 1.2	46.1 ± 2.3
	I	5.8 ± 0.3	6.0 ± 0.4
	T4	6.6 ± 0.2	6.3 ± 0.5
	T3	1.1 ± 0.2	1.0 ± 0.2
48 hr.	origin	5.2 ± 0.2	5.3 ± 0.3
	MIT	23.3 ± 0.6	22.6 ± 0.7
	DIT	43.1 ± 0.7	42.3 ± 1.4
	I	5.3 ± 0.2	4.9 ± 0.3
	T4	17.0 ± 0.3	17.2 ± 0.4
	T3	2.1 ± 0.2	2.2 ± 0.3

* Six hydrolysates were analysed at each time interval.

** Expressed as % total ^{131}I in the thyroid gland.

*** Mean ± S.E.

indicates that as good a quantitative estimate of the percentage distribution of ^{131}I in chromatograms of thyroid hydrolysates is achieved by densitometric scanning of radioautographs as by direct counting, if the conditions for exposure of the X-ray film mentioned above under Paper chromatography and radioautography are adhered to.

CHAPTER 2.

THE ESTIMATION OF IODINE IN THYROIDAL IODOAMINO ACIDS BY ALKALINE ASHING

INTRODUCTION.

The introduction of accurate techniques for the estimation of bound iodine in blood has greatly facilitated the growth of knowledge concerning the biochemical aspects of thyroid function. In the investigation of some of the more basic problems of intrathyroidal iodine metabolism, such as the kinetics of labelling of MIT and DIT and the pathway for the biosynthesis of T_3 and T_4, it is necessary to measure the absolute specific radioactivities of iodine in the various iodoamino acids. To achieve this, a quick, accurate and reliable method for the quantitative measurement of trace quantities of iodine is required.

In 1937 Sandell and Kolthoff published a method for the quantitative determination of extremely small amounts of iodide (0.01 µg. or less). In this method the iodide is measured by means of the catalytic effect it exerts in the reaction between Ce^{4+} and As^{3+}.

All procedures for protein-bound iodine (PBI), which measure the minute amount of iodine in T_3 and T_4 bound to serum proteins (4-8 µg./100 ml. serum in normal

subjects), employ the method of Sandell and Kolthoff for the colorimetric analysis of iodine. However, before the organic bound iodine can be measured colorimetrically, it must first be converted into inorganic iodide. Basically, two methods exist for the conversion of organic bound iodine to inorganic iodide. (1) acid digestion followed by distillation (Chaney, 1940) and (2) alkaline ashing (Salter and Mackay, 1944). Several modifications of the distillation method (Taurog and Chaikoff 1946, 1948; Barker, 1948; Connor, Swenson, Park, Gangloff, Lieberman and Curtis 1949; van Zyl, 1953; Spitzy and Lieb, 1956; van Zyl, 1961) and of the alkaline ashing method (Barker and Humphrey 1950; Grossman and Grossman 1955; Foss, Hankes and van Slyke, 1960) are being used. Elimination of the distillation of iodine from the acid digest by the use of chloric acid was introduced by Zak, Willard, Myers and Boyle(1952),and this method has been extensively used for the determination of iodine in studies of intrathyroidal iodine metabolism. In the method of Zak et. al. a small amount of chromic acid must be present in order to prevent losses of iodic acid, but the use of chromic acid has the disadvantage that it has a significant catalytic effect upon the ceric - arsenite reaction (Chaney, 1958).

In previous investigations of intrathyroidal iodine metabolism (van Zyl and Wilson, 1963) the distillation method (van Zyl, 1961) for the estimation of iodine in the thyroidal iodoamino acids was employed. Since the distillation step was tricky and laborious, and since a large number of samples had to be analysed in each experiment, it was subsequently decided to investigate the suitability of the alkaline ashing method in measuring stable iodine for specific activity measurements of the thyroidal iodoamino acids. As the iodoamino acids were purified by paper chromatography prior to iodine analysis, it is unlikely that they would contain non-volatile substances which might affect the reduction of Ce^{4+} by As^{3+} and iodide. For this reason it was thought possible to employ an alkaline ashing method and consequently eliminate the rather tedious digestion and distillation steps. There is also the added advantage that the recoveries of iodine are generally higher when determinations are made by alkaline ashing procedures. A disadvantage of the alkaline ashing method is that high concentrations of either Na^+ or K^+ are required to prevent losses of iodine by volatilization during incineration. Since K^+ and Na^+ depress the catalytic effect of iodide on the ceric -

arsenite reaction (Foss, Hankes and van Slyke, 1959, 1960), the present method, which uses KOH as the added alkali, was so designed that the lowest possible concentration of K^+ was used.

An accurate and reliable alkaline ashing method for the quantitative measurement of stable iodine in iodotyrosines and iodothyronines resolved from thyroid hydrolysates by paper chromatography is described. The reproducibility of the method, the recoveries of iodine and factors influencing the reduction of Ce^{4+} by As^{3+} and iodine were investigated.

The method described has been successfully used for the determination of absolute specific activities of thyroidal iodoamino acids in the study of the kinetics of labelling of MIT and DIT and in tracing the pathways of the biosynthesis of T_3 and T_4.

THE ALKALINE ASHING METHOD.

Cleaning of glassware.

All glassware was initially cleaned by washing with soap flakes and then soaked overnight in approximately 2N-KOH and rinsed several times with dichromate - sulphuric acid cleansing fluid, tap water, distrilled water and

"iodine-free water" (see below under Iodine-free water). The glassware used for iodine determinations was kept exclusivley for this purpose and immediately after use it was washed according to the procedure outlined above.

Unless the colorimetric tubes were grease-free erroneous results were obtained. Consequently to render these tubes clean and grease-free the following cleaning procedure was adopted. The tubes were rinsed several times with tap water, soaked overnight in 2N-KOH, rinsed with chromic acid cleansing fluid and tap water and then soaked for several hours in methanol and ammonia (3:I V/V). Thereafter the tubes were rinsed with hot water, scrubbed with a test tube brush (without using soap) and rinsed with tap, distilled and iodine-free water and subsequently dried in an oven at about 120^{o}.

REAGENTS.

All reagents were prepared with iodine-free water using "analytical" grade chemicals (B.D.H. "Analar" grade), except ceric ammonium sulphate where B.D.H. "Laboratory" grade was used.

Iodine-free water.

Iodine-free water was prepared by passing glass distilled water slowly through a demineralizer.

Only water registering one part per million or less on a control meter was used.

Arsenious acid reagent.

2.475g. As_2O_3 was dissolved in 25 ml. 1N-KOH with the aid of heat. This solution was added to approximately 600 ml. of water in a 1 litre volumetric flask. To this was added 54 ml. concentrated HCl followed by the gradual additions of 170 ml. concentrated H_2SO_4, the contents being well mixed and cooled before the final volume was adjusted to 1 litre at room temperature, by the addition of water.

Ceric ammonium sulphate reagent.

45 ml. of concentrated H_2SO_4 was gradually added to approximately 600 ml. of water in a 1 litre volumetric flask. 9.5g. of ceric ammonium sulphate, $Ce(SO_4)_2 \cdot 2(NH_4)_2SO_4 2H_2O$ was dissolved in this acid. After cooling to room temperature the solution was made up to the litre mark with water.

Potassium hydroxide (4N).

112.2g.KOH was dissolved in 500 ml. water.

Stock iodide solution. (100 µg. iodide/ml.)

130.8 mg. of KI, dried in an oven for 24 hr. at 120° and cooled in a desiccator, was dissolved in 1 litre of water, transferred to an amber bottle and stored in the refrigerator.

Working stock iodide solution. (0.20 µg. iodide/ml.)

2 ml. of the stock iodide solution was diluted to 1 litre with water.

Dilute iodide solutions for standard curve.

Solutions containing 0.01 - 0.08 µg. iodide/ 0.5 ml. were prepared by diluting 5, 10, 15, 20, 25, 30, 35 and 40 ml. of the working stock solution (0.20 µg. iodide/ml.) to 50 ml. with water.

PROCEDURE.

Preparation and drying of samples.

The portions of the developed chromatograms corresponding to inorganic iodide, MIT, DIT, T_3 and T_4 were cut into small pieces and put into incineration tubes. To each tube 0.5 ml. 4N-KOH was added carefully so that the paper was completely soaked. Thereafter the rack containing the tubes was transferred to a drying oven at 150°, the drying being completed in approximately 45 min.

Incineration.

The rack containing the dried iodine samples was transferred to a thermostatically controlled muffle furnace at 180° and within 20 - 30 min. the temperature was raised to 600°. The samples were incinerated in an upright position at that temperature for 1 hr.

During the heating-up period, the circulation of air in the furnace was reduced to a minimum in order to prevent any loss of iodine.

If the samples contain large amounts of paper frothing may occur during the heating-up period. This can be prevented by raising the temperature slowly (20 - 25 min.) from 180° to approximately 350° or by the addition, before drying of an antifoam solution (Dow Corning Antifoam). In the present procedure the former was adopted since it was considered desirable to avoid any unnecessary additions which could cause possible contamination.

Extraction of iodide from ash.

7 ml. of water was added to the ash in each tube and allowed to leach for 10 min., then without splashing the contents were stirred and allowed to stand for another 15 min., stirring at intervals before centrifuging the tubes for 15 min. at 2000 r.p.m.

The clear supernatant solution from each sample was transferred to a clean 15 x 125 mm. test tube, great care being taken not to transfer any carbon particles, as Foss, Hankes and van Slyke (1960) reported that carbon particles in the final solution accelerate the reaction between Ce^{4+} and As^{3+} thus causing errors in the colori-

metric analysis of iodine. The supernatant solution should be transferred shortly after centrifuging. It was found that if the supernatant remained on the ash low iodine values were obtained, particularly after standing for 24 hr.

Colorimetric analysis of iodide.

Since there is no maxium in the extinction curve of ceric sulphate solutions within the visible region, Beer's law is only obeyed in photometers which can provide almost monochromatic light. Consequently the present work has been done using a Beckman DU spectrophotometer. Thus, the procedure described, is for use with the Beckman DU spectrophotomer but it could be easily modified to suit any particular photometer supplying monochromatic light.

0.5 ml. portions of each clear supernatant solutions were measured in triplicate into specially cleaned 15 x 125 mm. test tubes (see above under Cleaning of glassware). 4.0 ml. of water was added to each iodine sample and then 1.0 ml. of the arsenious acid reagent was added slowly down the side of the tube in order to avoid evolution of CO_2. Iodine samples greater than 0.5 ml. can be read if the appropriate adjustment is made in the volume of water added, so that the final volume for the

colorimetric analysis remains at 6.5 ml. The volumes taken for the iodine samples and for the iodine standards should be exactly the same. The contents of each tube were thoroughly mixed without splashing by holding the test tube firmly in the middle between thumb and index finger and stroking the tube with the index finger of the other hand so as to set up a vortex.[1]

All the tubes were placed in a waterbath at 30°, allowing at least 10 min. for the system to reach the temperature of the waterbath.

In sequence at 30 sec. intervals, 1 ml. of ceric ammonium sulphate solution was added to the first set of triplicate iodine samples. The contents of each tube were thoroughly mixed and the tube returned to the waterbath. An interval of 4 min. was allowed to lapse before ceric ammonium sulphate solution was added to the next set of triplicate samples again at 30 sec. intervals.

This time-sequence was repeated until all the samples had received 1 ml. of ceric ammonium sulphate solution. Exactly 20 min. after the addition of the ceric ammonium sulphate, the extinction of each iodine sample was read at 420 mμ in a Beckman DU spectrophoto-

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1. A mechanical vortex stirrer can be used.

meter using 1 cm. cuvettes. (The time-sequence must be the same as that used during the addition of the ceric ammonium sulphate solution). Each cuvette was rinsed with a portion of the iodine sample of which the extinction value was required.

Preparation of the Standard Curve.

For each series of ash solutions a standard curve was prepared by plotting the logarithm of absorbance of the standard solutions against concentration of iodide.

0.5 ml. portions of the standard solutions containing 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 µg. iodide (see above under Iodide solutions for Standard curve) were measured out in triplicate into specially cleaned 15 x 125 mm. test tubes (see above under Cleaning of glassware).

To each test tube 3.5 ml. water, 0.5 ml. 0.285N-KOH solution (freshly prepared by diluting 3.75 ml. 4N-KOH to 50 ml. with water), and 1 ml. arsenious acid reagent were added. Standard solutions containing 0.00 µg. iodide (i.e. blanks) were prepared by measuring out 4.5 ml. water, 0.5 ml. of the KOH solution and 1 ml. arsenious acid reagent. The test tubes were warmed to 30°, 1 ml. ceric ammonium sulphate solution was added in the same sequence as directed for the ash solutions

and the extinction values were read after 20 min.

The extinction values of the standards were plotted against iodide concentration (µg.) on semi-logarithmic paper (the extinction values being plotted along the log. axis).

TABLE 2. shows a typical set of values for the construction of a standard curve as shown in Fig. 3. The iodide content of each of the ash solutions was determined by interpolation from this curve.

Preparation of blanks.

With each set of iodine analysis, three reagent blanks were prepared and subjected to the same procedure as the iodine samples. The blanks differed from the samples of unkown iodine content, only in that an untreated piece of Whatman No. 1 chromatography paper 6 x 1.5 cm. was used. The iodine content of Whatman No. 1 chromatography paper varied between 0.0004 - 0.001/9cm^2.

FACTORS INFLUENCING THE ACCURACY OF THE RESULTS.

1. Iodine recoveries after drying and incineration.

(a) With ^{131}I-iodide.

Sufficient carrier-free Na^{131}I to give count rates of 100 - 2000 counts/sec. was applied to strips of Whatman No. 1 chromatography paper 6 x 1.5 cm. The

TABLE 2.

Data for the construction of a typical standard curve.

Sample	µg. Iodide	Extinction *
Water blank	0.00	0.720
Working standards	0.01	0.562
	0.02	0.441
	0.03	0.346
	0.04	0.276
	0.05	0.217
	0.06	0.171
	0.07	0.135
	0.08	0.106

* Each extinction value represents the mean of triplicate analyses.

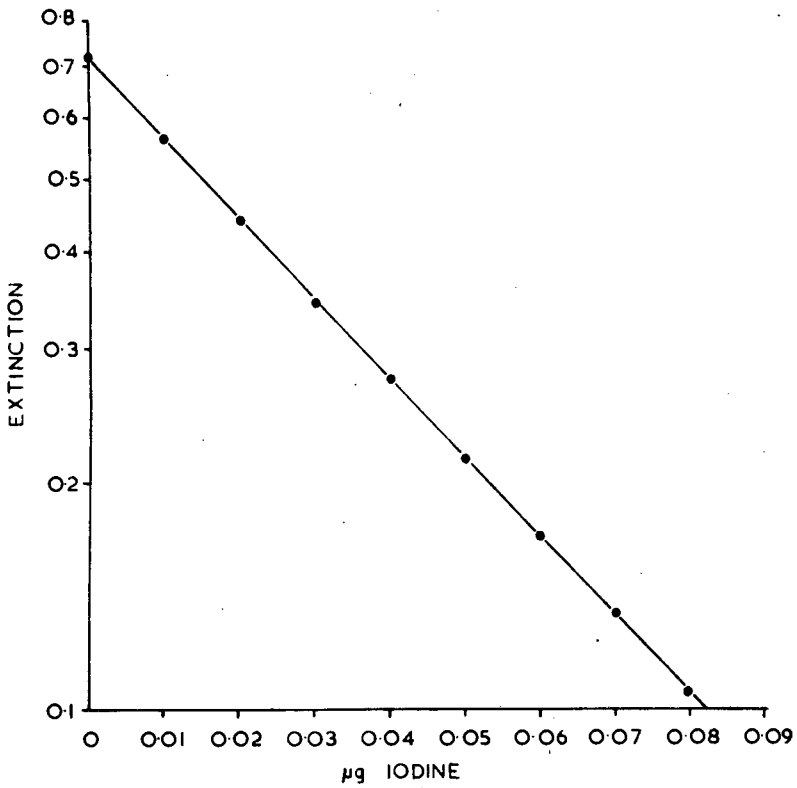

Fig. 3. Standard curve for the determination of iodide.

counting time was chosen so as to give an accuracy of within 0.5%. 0.5 ml. 4N-KOH was added to each sample which was then dried for 1 hr. at 150° and incinerated as previously described under Procedure.

TABLE 3. indicates that a loss of only 0.3% of inorganic ^{131}I occurred during the drying period and that the mean percentage recovery after incineration was 98.81 ± 0.60% (mean ± S.D.)

(b) With ^{131}I iodotyrosines and ^{131}I iodothyronines.

Biosynthetically ^{131}I-labelled MIT, DIT, T_3 and T_4 were purified by ascending paper chromatography in BDA and BAW and identified by autoradiography. The portions of the chromatograms corresponding to these iodoamino acids were cut out and counted. Enough counts were collected for a statistical accuracy of 1% or better. After the addition of 0.5 ml. 4N-KOH each sample was dried and incinerated as described under Procedure.

TABLE 3. indicates that only very small losses of iodotyrosines and iodothyronines occurred during drying (0.2 - 0.4%) and that the percentage recovery after incineration ranged from 98 - 99%.

2. The optimal concentration of Ce^{4+}.

For maximum accuracy in colorimetric analysis using the Beckman DU spectrophotometer, the extinction

TABLE 3.

The recovery of radioactive iodine after drying and after incineration.

Substance	Number of determinations	Percentage of added ^{131}I recovered after drying	Percentage of added ^{131}I recovered after incineration
$Na^{131}I$	11	99.69 ± 0.21*	98.81 ± 0.60
^{131}I Mono-iodotyrosine	16	99.81 ± 0.30	99.49 ± 1.26
^{131}I Di-iodotyrosine	18	99.64 ± 0.27	98.72 ± 0.46
^{131}I Tri-iodothyronine	18	99.82 ± 0.36	98.76 ± 0.63
^{131}I Thyroxine	18	99.75 ± 0.27	98.89 ± 1.23

* Mean ± S.D.

values of the iodine samples should be within the limits of 0.80 and 0.10. Accordingly it would be desirable to have the extinction values for the maximum amount of iodine for which the method is intended close to 0.10 and the extinction value for a sample containing no iodine (i.e. a water blank) in the vicinity of 0.70 - 0.80. In this particular work the method was designed to analyse to a limit of 0.08 µg. iodide.

In order to determine the optimal concentration of ceric ammonium sulphate for maximum accuracy, the extinction values of water blanks and of samples containing 0.08 µg. iodide were read with increasing concentrations of ceric ammonium sulphate. Since it has been common practice to control the colorimetric reaction at a temperature of 37° and to read the decolourization after an interval of 20 min., these conditions were originally selected.

The initial concentration[2] of As^{3+} used was the same as that of the alkaline ashing method of Foss, Hankes, and van Slyke (1960) for protein-bound iodine i.e. 0.0077N. The initial concentration of K^{+} was 0.022N.

---

2. The initial concentration represents at zero time the concentration for a reagent during the colorimetric analysis (concentration for a reagent immediately after ceric ammonium sulphate has been added).

Fig. 4. indicates the change in the extinction values for a water blank and a sample containing 0.08 µg. iodide with increasing concentration of ceric ammonium sulphate at 37°. At this temperature there was no suitable concentration of ceric ammonium sulphate which would give the extinction values within the range required for maximum accuracy. The reaction proceeded far too rapidly with the result that the extinction value for the 0.08 µg. iodide sample was considerably below 0.10 when the water blank was within the range 0.70 - 0.80. However, when the temperature was reduced to 30° (see below under Effect of temperature on the reduction of Ce^{4+} by As^{3+} and iodine), the extinction values of the water blank, and the 0.08 µg. iodide sample were within the limits required for maximum accuracy over a fairly wide range of initial ceric ammonium sulphate concentrations (Fig. 5.).

An initial Ce^{4+} concentration of 0.0023N was selected for the colorimetric analysis of iodine. This concentration of Ce^{4+} gave extinction readings for the limits for which the method was intended and well within the desired range.

3. <u>The effect of temperature on the reduction of Ce^{4+} by As^{3+} and iodine</u>.

Temperature has a profound effect on the reation rate of the ceric-arsenite reaction. For every

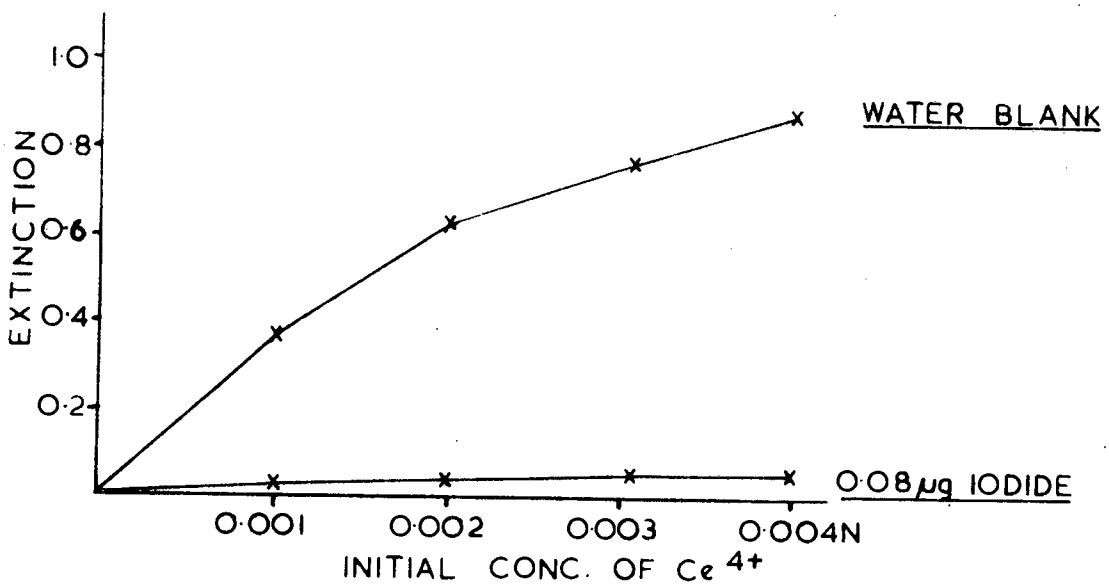

Fig. 4. The effect of different initial concentrations of Ce^{4+} on the reaction between Ce^{4+} and As^{3+} catalysed by iodine. Temperature 37°.

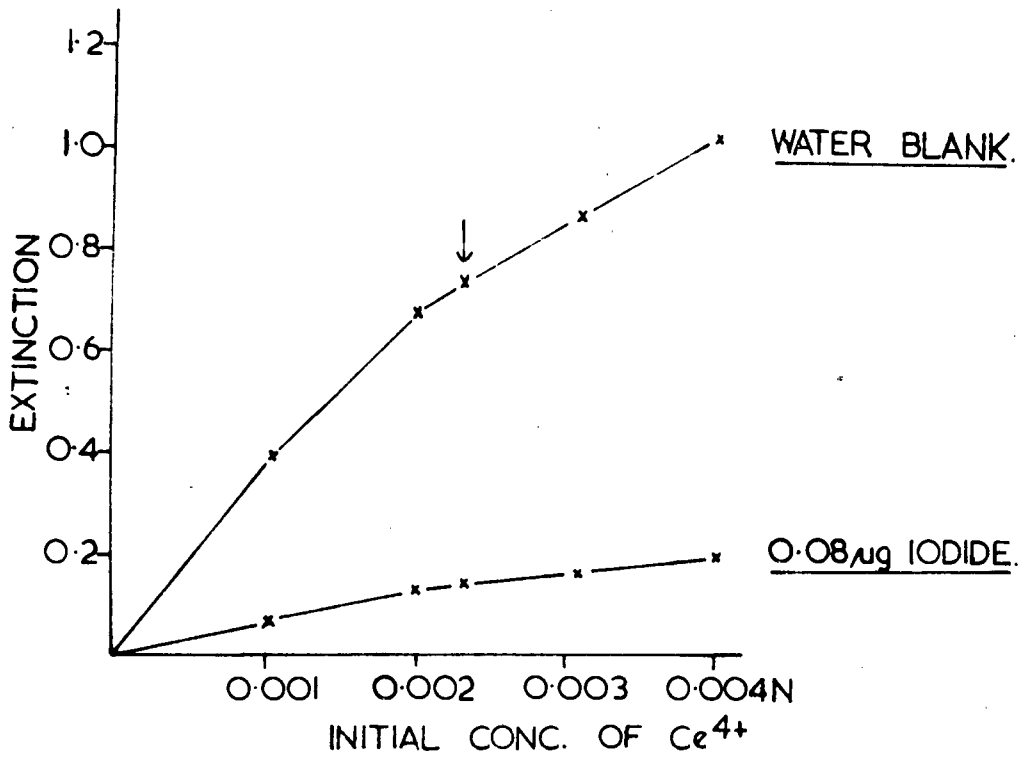

Fig. 5. The effect of different initial concentrations of Ce^{4+} on the reaction between Ce^{4+} and As^{3+} catalysed by iodine. Temperature 30°.

10^{o} drop in temperature the reaction rate is approximately halved. The temperature coefficient for a 10^{o} interval is 1.72 in the temperature range 17^{o} - 37^{o} (Moran, 1952).

Theoretically any temperature may be selected for assay purposes providing it remains constant during the period of the colorimetric analysis. Most workers recommend 37^{o} as a convenient working temperature. However, as already pointed out, for the conditions selected, the reaction was too rapid at 37^{o} for all initial concentrations of Ce^{4+}. For this reason, the extinction readings of a water blank and a sample containing 0.08 µg. iodide were measured with increasing initial concentrations of Ce^{4+} at varying temperatures (Fig. 6.). Reducing the temperature from 37^{o} to 30^{o} brings the extinction readings within the limits of maximum accuracy. Lowering the temperature below 30^{o} tends only to decrease the range of initial Ce^{4+} concentration which will give the desired extinction values. In the routine procedure followed, the reaction was performed at 30^{o} with an initial Ce^{4+} concentration of 0.0023N.

Effect of K^{+} on the reduction of Ce^{4+} by As^{3+} and iodine.

In all the well recognised alkaline ashing procedures for the determination of protein-bound iodine

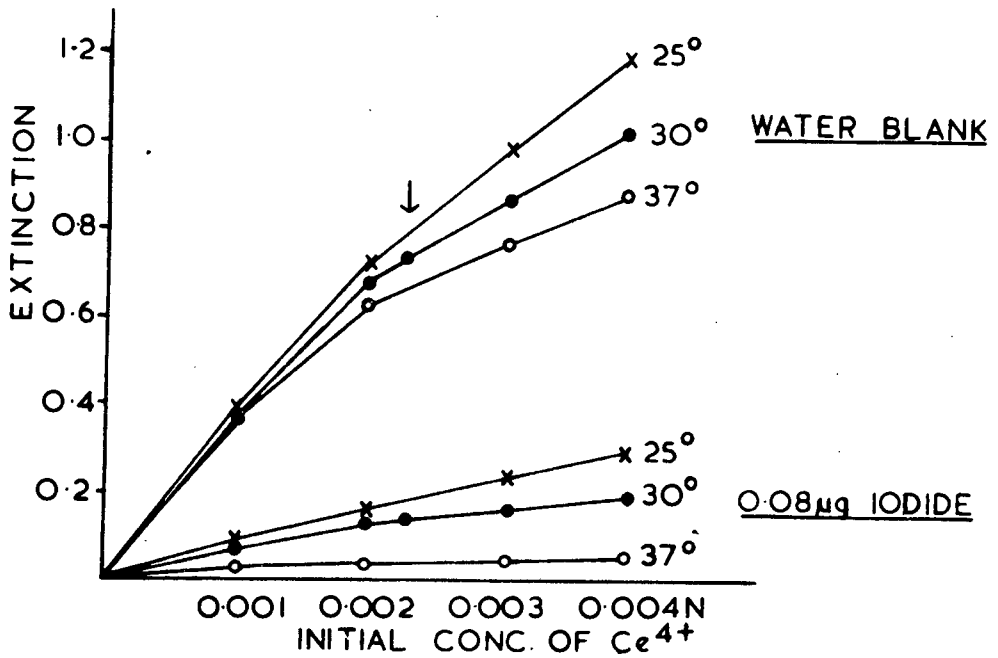

Fig. 6. The effects of temperature and of different initial concentrations of Ce^{4+} on the reaction between Ce^{4+} and As^{3+} catalysed by iodine. The arrow indicates the zero time concentration of Ce^{4+} used in the routine procedure.

now available (Barker and Humphrey, 1950; Barker, Humphrey and Soley 1951; Grossman and Grossman 1955; Foss, Hankes and van Slyke, 1960) the initial concentration of K^+(or Na^+) is high i.e. within the range of 0.120 - 0.170N. However, it has been pointed out by Foss, Hankes and van Slyke (1959, 1960) that K^+, Na^+ and Li^+ depress the catalytic effect of iodine in the ceric-arsenite reaction, the inhibiting effect being greater for K^+ than for either Na^+ or Li^+. Consequently it was important to study the effect of K^+ on the conditions selected for the present method, so that the maxium sensitivity in the colorimetric analysis of iodine could be attained.

By raising the initial concentration of K^+ to 0.3N, the reaction was inhibited and the extinction values were increased to a marked extent, especially beyond 0.1N (Fig. 7.). An increase in the initial concentration of K^+ into the range 0.12-0.17N caused a reduction of approximately 35% in the sensitivity of the ceric - arsenite reaction. As a result of the diminution in the sensitivity of the ceric - arsenite reaction, a low initial concentration of K^+ would be advantageous and consequently in the procedure adopted, the lowest possible initial concentration of K^+ (0.022N) was used.

Although at this concentration the inhibiting effect of K^+ is very small, the concentration of K^+ is

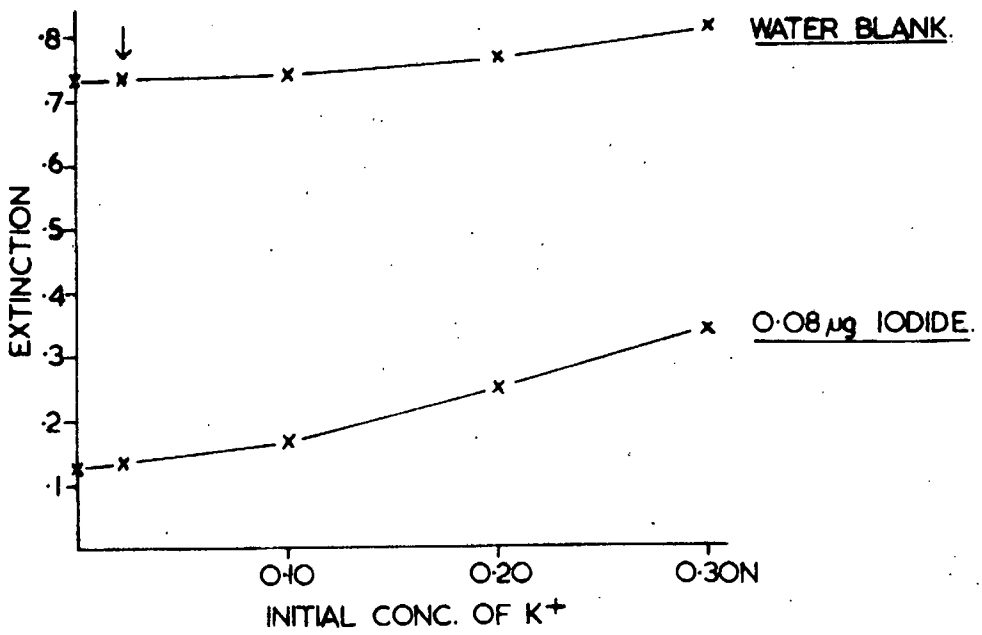

Fig. 7. The effect of different concentrations of K^+ on the reaction between Ce^{4+} and As^{3+} catalysed by iodine. The arrow indicates the zero time concentration of K^+ used in the routine procedure.

also such that it is much greater than that which would be expected in any of the iodine samples to be analysed.

PRECISION OF THE METHOD.

(a) Reproducibility in analyses of standard solutions.

Nine samples each containing 0.025 µg. iodide and nine samples each containing 0.05 µg. iodide were prepared in triplicate as outlined under the directions for Preparation of the standard curve. The standard deviation from the mean of the extinctions of 9 triplicate readings was equivalent to ± 0.0002 µg. iodide for samples containing 0.025 µg. iodide and ± 0.0003 µg. iodide for the 0.05 µg. iodide samples (TABLE 4.).

(b) Reproducibility in analyses of ash solutions.

0.05 µg. iodide samples were applied to strips of Whatman No. 1 chromatography paper 6 x 1.5 cm. Seven such samples were analysed in triplicate as described under Procedure. The standard deviation from the mean of the extinctions was equivalent to ± 0.0005 µg. iodide (TABLE 5.).

RECOVERIES.

(a) Potassium iodide.

0.1 ml. samples containing 0.05 µg. iodide were applied to strips of 6 x 1.5 cm. Whatman No. 1 chromatography paper. The iodide samples together with

TABLE 4.

Reproducibility of extinction values measured in 9 individual standard solutions of 0.025 µg. and of 0.050 iodide respectively.

Iodide µg.	Sample No.	Extinction*	Iodide µg.	Sample No.	Extinction*
0.025	1	0.380	0.050	1	0.221
	2	0.383		2	0.216
	3	0.382		3	0.217
	4	0.385		4	0.220
	5	0.382		5	0.218
	6	0.381		6	0.213
	7	0.382		7	0.218
	8	0.383		8	0.219
	9	0.384		9	0.217
	MEAN	0.382		MEAN	0.218
	Standard Deviation	0.0017		Standard Deviation	0.0025

* Each extinction value represents the mean triplicate analyses.

TABLE 5.

Reproducibility of extinction values measured in seven individual ash solutions.

µg. Iodide	Sample No.	Extinction *
0.050	1	0.214
	2	0.217
	3	0.217
	4	0.214
	5	0.215
	6	0.219
	7	0.216
	MEAN	0.216
	Standard Deviation	± 0.0031

*Each extinction value represents the mean of triplicate analyses.

blanks were analysed as outlined under Procedure. Simultaneously, control samples containing 0.05 µg. iodide were analysed as described under Preparation of standard curve. TABLE 6. shows that the recovery of iodine added as KI was 98.0 ± 0.58% (mean ± S.D.).

(b) Iodotyrosines and iodothyronines.

The purity of DIT, T_3 and T_4 (donated by Glaxo Ltd.) was ascertained by determining their molar extinction coefficients and by comparing with those as determined by Gemmill (1955). 5 - 10 mg. of each substance was dissolved in 100 ml. 0.05N-KOH. The weight taken of each compound was such as to obtain an absorbance value close to 0.7 at the maximum occurring in the wavelength range 300 - 330 mµ.

The absorption spectrum for each compound was determined in 0.04N-KOH at room temperature (23 ± 2°) for wavelengths 250 - 400 mµ. A Beckman DB recording spectrophotometer with 1 cm. quartz cuvettes was used. A cuvette filled with 0.04N-KOH was used to set the spectrophotometer at zero extinction.

In all cases the molar extinction (E expt.) was calculated according to the formula:

$$E\ expt. = \frac{A \times M}{L \times C}$$

TABLE 6.

Recovery after incineration of stable iodide added to Whatman No.1 chromatography paper.

	Sample No.	Iodine added µg.	Extinction*	Iodine recovered after incineration µg.	% Recovery
Incinerated Blanks	1	0.00	0.708	0.0008	-
	2	0.00	0.711	0.0005	-
	3	0.00	0.710	0.0006	-
	MEAN			0.0006	
Incinerated Samples	1	0.05	0.214	0.0493 **	98.6
	2	0.05	0.217	0.0489	97.8
	3	0.05	0.217	0.0489	97.8
	4	0.05	0.214	0.0493	98.6
	5	0.05	0.215	0.0492	98.4
	6	0.05	0.219	0.0486	97.2
	7	0.05	0.216	0.0490	98.0
	MEAN				98.0
	Standard Deviation				± 0.58

* Each extinction value represents the mean of triplicate analyses.

** Correction for the blank value has been made.

where A is the maxium absorbance, M the molecular weight of the compound, L (cm.) the length of the cuvette and C (g./litre) the concentration. The percentage purity (TABLE 7.) was given by the equation $\frac{E_{expt.}}{E'} \times 100$ where E' is the molar extinction value of the compound as determined by Gemmill (1955).

5 ml. of each of the solutions used to determine the purity were diluted to 100 ml. with iodine free water. Of each of these diluted solutions 0.1 ml. was applied to strips of Whatman No. 1 chromatography 6 x 1.5 cm. using an "Agla" syring. The iodine in each sample was estimated as outlined under Procedure.

Using the experimentally determined purity, the amount of iodine in each sample of DIT, T_3 and T_4 applied to the chromatography paper was calculated. These values were used to estimate the percentage recoveries of iodine in DIT, T_3 and T_4, after incineration. TABLE 8. shows the percentage recovery for the individual thyroidal iodoamino acids.

Based on the recoveries obtained for iodide, DIT, T_3 and T_4, the overall percentage recovery for the method reported is 97.93 $\pm$ 0.89% (mean $\pm$ S.D.).

TABLE 7.

Determination of the percentage purity of various iodotyrosines and iodothyronines used for recovery in the dry ashing procedure, employing molar extinction coefficients.

Substance	Molar Extinction* (in 0.04N-KOH)	Experimentally determined Molar extinction (in 0.04N-KOH)	% Purity
3,5 Di-iodo-L-tyrosine.$2H_2O$	5916	5840	98.72
		5850	98.88
		5847	98.83
3,5,3'Tri-iodo-L-thyronine	4658	4450	95.53
		4442	95.36
		4450	95.53
Na-L-Thyroxine .$5H_2O$	6207	6160	99.24
		6160	99.24
		6158	99.21

* Values quoted by C.L. Gemmill (1955).

TABLE 8.

Recovery after incineration of stable iodine in iodotyrosines and iodothyronines added to Whatman No. 1 chromatography paper.

	Sample No.	Iodine added µg.	Extinction *	Iodine recovered after incineration ** µg.	% Recovery
DIT	1	0.0391	0.283	0.0380	97.19
	2	0.0391	0.281	0.0384	98.21
	3	0.0391	0.280	0.0385	98.47
	4	0.0222	0.408	0.0219	98.65
	5	0.0222	0.410	0.0217	97.75
	6	0.0222	0.410	0.0217	97.75
	7	0.0300	0.347	0.0294	98.00
	8	0.0300	0.347	0.0294	98.00
	9	0.0300	0.346	0.0296	98.67
	10	0.0300	0.348	0.0293	97.77
	MEAN				98.05
	Standard Deviation				± 0.44
T_3	1	0.0337	0.317	0.0334	99.11
	2	0.0337	0.320	0.0330	97.92
	3	0.0337	0.321	0.0328	97.33
	4	0.0337	0.319	0.0321	98.22
	5	0.0337	0.322	0.0327	97.03
	6	0.0337	0.318	0.0333	98.81
	MEAN				98.07
	Standard Deviation				± 0.89
T_4	1	0.0284	0.363	0.0279	98.24
	2	0.0284	0.367	0.0276	97.18
	3	0.0284	0.364	0.0278	97.89
	4	0.0284	0.366	0.0277	97.54
	5	0.0279	0.367	0.0276	98.92
	6	0.0279	0.370	0.0273	97.85
	7	0.0279	0.369	0.0274	98.21
	8	0.0279	0.372	0.0272	97.49
	9	0.0279	0.368	0.0275	98.57
	MEAN				97.99
	Standard Deviation				± 0.61

* Each extinction value represents the mean of triplicate analyses.
** Correction for blank value has been made.

CHAPTER 3.

IODINE METABOLISM IN RATS ON A NORMAL IODINE DIET.

INTRODUCTION.

In the present study an investigation of iodine metabolism in rats fed a normal iodine diet involved the following: (1) A kinetic study in which a three-compartment system was used to determine the rates of thyroidal ^{131}I uptake and secretion, and the rate of peripheral degradation of endogenously synthesized organically bound ^{131}I. (2) A study of the kinetics of labelling of the various thyroidal iodoamino acids using absolute specific activities and the distribution of ^{131}I between the iodoamino acids. For the second study, rats were killed at various intervals (a)2hr.-168 hr. and (b) at 30 sec. - 90 min. after the injection of ^{131}I.

PART 1. THE KINETICS OF TOTAL IODINE METABOLISM.

METHODS.

(For detailed descriptions of the procedures used refer to Chapter 1.)

Male Sprague-Dawley rats weighing 330 $\pm$ 15g. were used. The animals were fed a commercial pellet diet containing 30 μg. iodide/100g. and had free access to tap water. The daily intake of iodine was estimated at

5-6 µg. per rat. Seventy-four rats were injected intraperitoneally with 34.4 µc. carrier-free $Na^{131}I$. Of these, 30 rats were immediately placed separately in metabolism cages which permitted the separate collection of urine and faeces. At each period of 0.5, 1, 2, 4 and 6 hr. four rats were killed, the thyroid glands were removed, blood samples were taken and the urine and faeces were collected.

The animals which were not placed in the metabolism cages were killed at intervals from 8-216 hr., four animals being killed at each interval. Simultaneously at each time of killing, urine and faeces were collected from the remaining ten animals in the metabolism cages and counted.

Collection and Analysis of Specimens.

(a) Thyroid Glands.

The thyroids were dissected and the uptake of ^{131}I was measured using a ring counter, previously described.

(b) Blood.

Blood was collected by cardiac puncture, allowed to clot and centrifuged at 1200 r.p.m. for 15 min. Serum samples (1 ml.) were assayed for their ^{131}I content. All determinations were made in a well-type scintillation counter and counted to a statistical accuracy of at least

2%. In order to determine the percentage of organic and inorganic radioiodine in the serum, 1 ml. serum samples were fractionated on 1.5 cm. "Ioresin" columns. The resin was eluted twice with 1 ml. water so that the total eluent collected was 3 ml. The eluent ($PBI^{131}I$) was counted and the difference between the activity in the sample applied to the resin and that in the eluent was the fraction of the activity in the serum present as ^{131}I iodide. In several instances the resin was counted as a check and in all cases was found to agree within 1.5% of the ^{131}I iodide value obtained by difference.

The ^{131}I fractions present in the sera were expressed both in µc. and as a percentage of the injected dose by counting against three standard samples of 3 ml. each and containing 0.0344 µc. of the initial activity injected into each rat.

Sera from blood samples taken from appropriate groups of animals during the experiment were analysed for total ^{127}I and $PB^{127}I$ by the ashing method of Grossman and Grossman (1955). The circulating ^{127}I iodide was calculated from the difference between the total ^{127}I and the $PB^{127}I$.

(c) Urine and faeces.

Complete urine and faecal collections were made during each interval from the rats in the metabolism cages.

At the end of each interval the funnels of the cages were rinsed thoroughly and the washings were added to the urine collected during that interval. All urine specimens were made up to 30 ml. and counted.

Rats which were killed during the period 0.5-6 hr. after the administration of ^{131}I were also housed in metabolism cages and at the appropriate time each rat was gassed in the metabolism cage by enclosing the cage in a polythene bag. The urine present in the bladder at the time of killing was added to that collected during the interval.

THEORETICAL CONSIDERATIONS.

Many of the observations in thyroid physiology may be explained on the hypothesis that iodine is present within the body in several identifiable compartments (Oddie, 1949; Brownell, 1951; Riggs, 1952; Berson and Yalow, 1954; Oddie, Meschan and Wortham, 1955.)

Brownell (1951) developed a particularly useful mathematical analysis of iodine metabolism in man which was based on a three-compartment system.

The first compartment represents inorganic iodine in the plasma, extracellular fluid, and in the thyroid. The second compartment represents the organic iodine (consisting mainly of T_4 and T_3) in the blood and other extracellular fluid.

Lastly, the third compartment represents the organic iodine in the thyroid which is present as MIT, DIT, T_3 and T_4. However, practical analysis of data from even such a simple model as the three-compartment system of iodine metabolism proved to be a formidable task.

The mathematical treatment of the present investigation on rats is based on the model illustrated in Fig. 8 . After injection, the ^{131}I is rapidly distributed between the iodide pools of the blood and tissue fluids. Clearance of the total iodide pool is effected by the trapping mechanism of the thyroid and by excretion in the urine. The ^{131}I trapped by the thyroid is organically bound and released slowly into the blood. In the peripheral tissues this organic iodide undergoes metabolic degradation, the principal end-product being iodide which is partly excreted in the urine and partly reaccumulated by the thyroid.

For the purpose of simplifying the mathematical analysis the following assumptions have been made:

1. The administered ^{131}I iodide rapidly diffuses throughout the blood compartment (V_2) and reaches isotopic equilibrium within a few min. with the stable iodide already present.

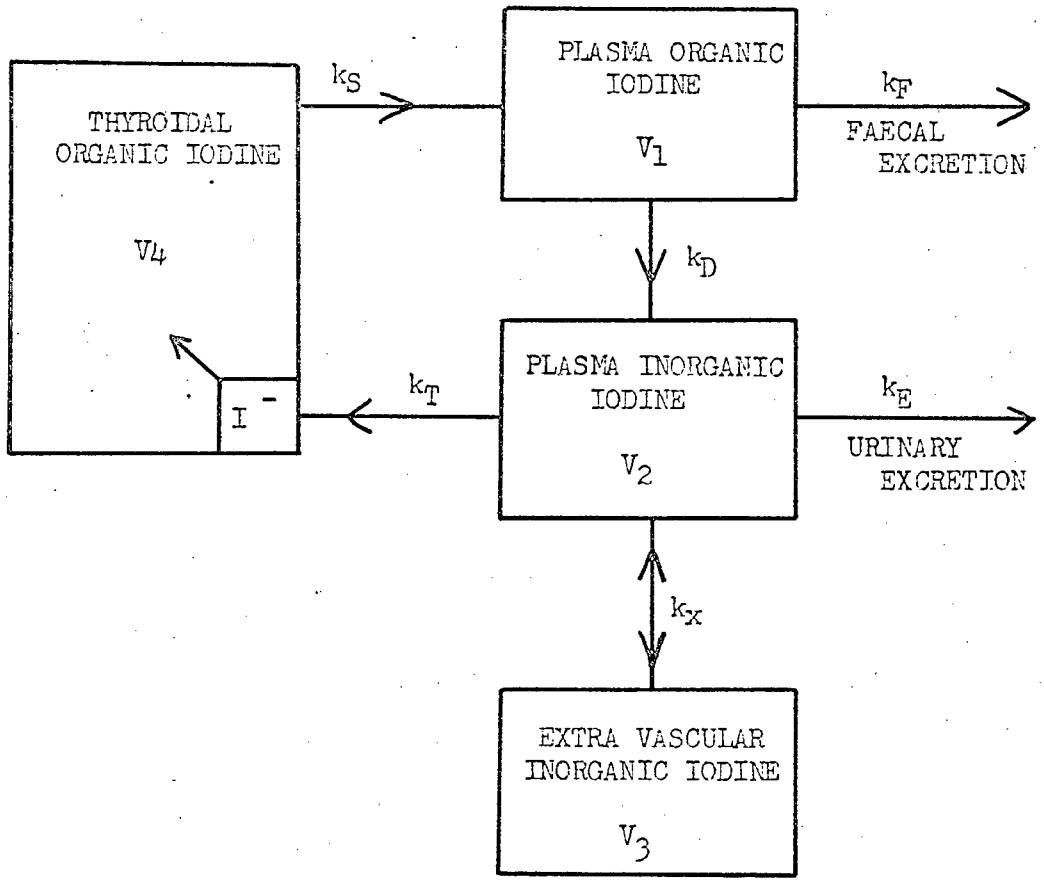

Fig. 8. Assumed model of iodine metabolism.

2. The ^{131}I iodide in the blood diffuses gradually into the tissue fluid compartment (V_3) at a rate k_x and comes into isotopic equilibrium with the stable iodide in this compartment. It is assumed that the diffusion of ^{131}I iodide from the blood into the tissue fluids is essentially complete after 2 hr. Consequently, after 2 hr. the blood and tissue fluids can be considered as a single compartment of volume ($V_2 + V_3$) ml. having the same concentration of ^{131}I. The volume ($V_2 + V_3$) is known as the iodide space.

3. The thyroidal iodine is regarded as being entirely organic since less than 1% of the total thyroidal iodine is inorganic.

4. The thyroid gland has been considered to act as a single iodine compartment and the ^{131}I released from the thyroid is regarded as being entirely in the form of T_3 and T_4.

5. Only inorganic iodide is considered to be excreted in the urine.

(a) <u>The loss of ^{131}I from the iodide space and determination of the iodide space</u>.

Referring to the model illustrated in Fig. 8. consider, at early intervals after the injection of a single tracer dose of ^{131}I iodide, the removal of ^{131}I from the iodide space ($V_2 + V_3$) by the accumulation

in the thyroid at a rate of k_T and by excretion in the urine at the rate of k_E.

The amount of ^{131}I iodide (At) remaining in the iodide space at time t can be derived from the equation,

$$A_t = Ao\ e^{-(k_T + k_E)t} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where Ao = initial activity of ^{131}I injected.

Similarly, the concentration of the ^{131}I iodide (I_t) remaining in the iodide space at time t is,

$$I_t = \frac{1}{(V_2 + V_3)}\ e^{-(k_T + k_E)t} \quad \ldots\ldots\ldots\ldots (2)$$

Since after 2 hr. it is assumed that most of the ^{131}I has uniformly mixed throughout the iodide space, the constant $(k_T + k_E)$ may be obtained from the semi-logarithmic plot of serum ^{131}I iodide concentration as a function of time for intervals greater than 2 hr. after injection (the concentrations are plotted along the logarithmic axis). The intercept of the logarithmic axis gives the value of $\frac{1}{(V_2 + V_3)}$ and consequently the volume of iodide space $(V_2 + V_3)$ can be calculated.

(b) <u>Thyroidal uptake and release of ^{131}I</u>.

If it is assumed during early intervals after injection of ^{131}I that all the ^{131}I accumulated by the thyroid is stored and that only negligible amounts of ^{131}I are returned to the iodide space or secreted as

hormone, then the quantity of ^{131}I (b_t) accumulated by the thyroid at time t is given by the equation,

$$b_t = \frac{k_T}{k_T + k_E} (A_o - A_t) \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Substituting the value of A_t from equation (1),

$$b_t = \frac{k_T}{k_T + k_E} A_o \left[1 - e^{-(k_T + k_E)t} \right] \quad \ldots\ldots\ldots (4)$$

At later intervals allowance for the secretion of ^{131}I as hormone has to be made. Hence the fraction of $^{131}I(b_{t_1})$ which has accumulated in the gland at later intervals can be derived from the expression,

$$b_{t_1} = \frac{k_T}{k_T + k_E} A_o \left[1 - e^{-(k_T + k_E)t} \right] e^{-k_s t} \quad \ldots (5)$$

where k_s = the fractional rate of release of ^{131}I from the thyroid.

In addition, most of the ^{131}I released by the thyroid is subjected to metabolic degradation in the peripheral tissues, the principal end-product being ^{131}I iodide. Part of this ^{131}I iodide is reaccumulated by the thyroid and the rest is excreted in the urine. In order to allow for the reutilization of ^{131}I iodide from the hormone secreted, equation (5) must be modified as follows;

$$b_{t_1} = \frac{k_T}{k_T + k_E} A_o \left[1-e^{-(k_T + k_E)t} \right] e^{-k_s t} + F \ldots\ldots(6)$$

Where F = fraction of ^{131}I released as hormone which is reaccumulated by the thyroid.

Since the measured value of the thyroid uptake at later intervals should be given by equation (6), it is theoretically possible to calculate the fraction of ^{131}I reutilized by the thyroid simply by subtracting the value of b_{t_1} calculated from equation (5) from the measured uptake.

The fractional rate of release ^{131}I from the thyroid (k_s) is obtained from the slope of the semi-logarithmic curve for the ^{131}I loss occurring from the thyroid after the maximum uptake. However, the rate of loss of ^{131}I measured from this curve represents the difference between the actual loss of ^{131}I as hormone and the uptake of ^{131}I derived from the degraded hormone. Consequently the actual ^{131}I release constant (k'_s) will be obtained from the equation;

$$k'_s = \frac{k_s}{(1 - F)} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(7)$$

(c) Excretion of ^{131}I.

At early intervals the cumulative excretion of ^{131}I in the urine (U_t) between zero time and time t is derived as follows:

$$U_t = \frac{k_E}{k_T + k_E} A_o \left[1 - e^{-(k_T + k_E)t} \right] \quad \ldots\ldots(8)$$

Assuming that at the later intervals the extra-thyroidal ^{131}I iodide remaining from the initial injection is negligible, the rate of excretion of ^{131}I iodide in the urine (k'_E) will equal the release of ^{131}I from the thyroid less the amount of ^{131}I iodide reaccumulated by the thyroid. i.e.,

$$k'_E = k_S (1 - F) \quad \ldots\ldots(9)$$

(d) Concentration of organic ^{131}I in the serum.

If hormonal secretion is proportional to the concentration of ^{131}I-labelled T_4 and T_3 in the gland (H) and if the interval after injection of ^{131}I is short enough so that no organic ^{131}I is degraded or excreted, the concentration of $PB^{131}I$ (P) at time t is given by,

$$P = H \left[1 - e^{-k_o t} \right] \quad \ldots\ldots(10)$$

Where k_o = rate of appearance of $PB^{131}I$ in the serum.

At intervals after the maximum $PB^{131}I$ concentration is reached, the rate of decrease in $PB^{131}I$ is equal to the rate of release of ^{131}I from the gland (k_s).

PRACTICAL APPLICATIONS OF THE MODEL.

The object of this investigation was to determine the extent to which the simple model illustrated in Fig. 8. explains the metabolism of iodine in normal rats.

Wherever possible theoretically derived values were compared with the experimental values.

(a) Estimation of the iodide space and of the constants k_T and k_E.

It can be seen from equation (2) that for intervals greater than 2 hr. after injection of ^{131}I, the semi-logarithmic plot of the percentage of the administered ^{131}I present in the serum as a function of time will yield a straight line with a slope equal to $(k_T + k_E)$. Extrapolation of this line to zero time will give the value $\frac{100}{(V_2 + V_3)}$.

The data in TABLE 9. were used to obtain the semi-logarithmic plot of the variation in serum ^{131}I iodide concentration with time illustrated in Fig. 9. From the curve the constant $(k_T + k_E)$ and the iodide space were found to be 0.139/hr. and 244 ml. respectively.

Similarly, equations (4) and (8) indicate that the constant $(k_T + k_E)$ can be obtained from the renal excretion of ^{131}I and the thyroidal uptake of ^{131}I. The data in TABLE 9. and TABLE 10. were used to obtain the semi-logarithmic curves of the concentration of ^{131}I in the thyroid and the urine as a function of time illustrated in Fig. 10(a) and Fig. 10(b).

TABLE 9.

Concentration of ^{131}I in the thyroid gland and serum of rats fed a diet adequate in iodine (30 µg.^{127}I/100g.) and killed 0.5 - 216 hr. after injection of ^{131}I.

Time after ^{131}I hr.	Thyroid ^{131}I uptake *		Serum ^{131}I *			
			Iodide/ml.		PBI/ml.	
	µc.	% dose	µc. x 10-2	% dose	µc. x 10-3	% dose
0.5	0.71	2.06	27.287	0.7936	0.351	0.1022
1	0.93	2.70	15.533	0.4506	0.475	0.1381
2	1.34	3.90	12.061	0.3488	0.647	0.1883
4	2.89	8.40	8.604	0.2500	1.010	0.2935
6	3.37	9.80	6.483	0.1890	1.930	0.5609
8	3.92	11.40	4.296	0.1250	1.680	0.4884
12	5.15	14.98	2.908	0.0843	2.875	0.8357
15	5.55	16.13	1.800	0.0523	3.943	1.1470
18	5.68	16.50	1.233	0.0360	6.639	1.9305
24	5.36	15.60	0.362	0.0105	5.790	1.6821
36	5.46	15.89	0.299	0.0087	7.041	2.0446
48	5.36	15.60	0.162	0.0047	7.200	2.0901
72	4.65	13.52	0.151	0.0044	6.405	1.8636
96	-	-	-	-	-	-
120	3.72	10.81	0.080	0.0023	4.720	1.3733
144	-	-	-	-	-	-
168	2.73	7.93	0.055	0.0016	3.362	0.9770
192	-	-	-	-	-	-
216	2.07	6.02	0.045	0.0013	2.860	0.8319

* Each value represents the mean of 4 rats.

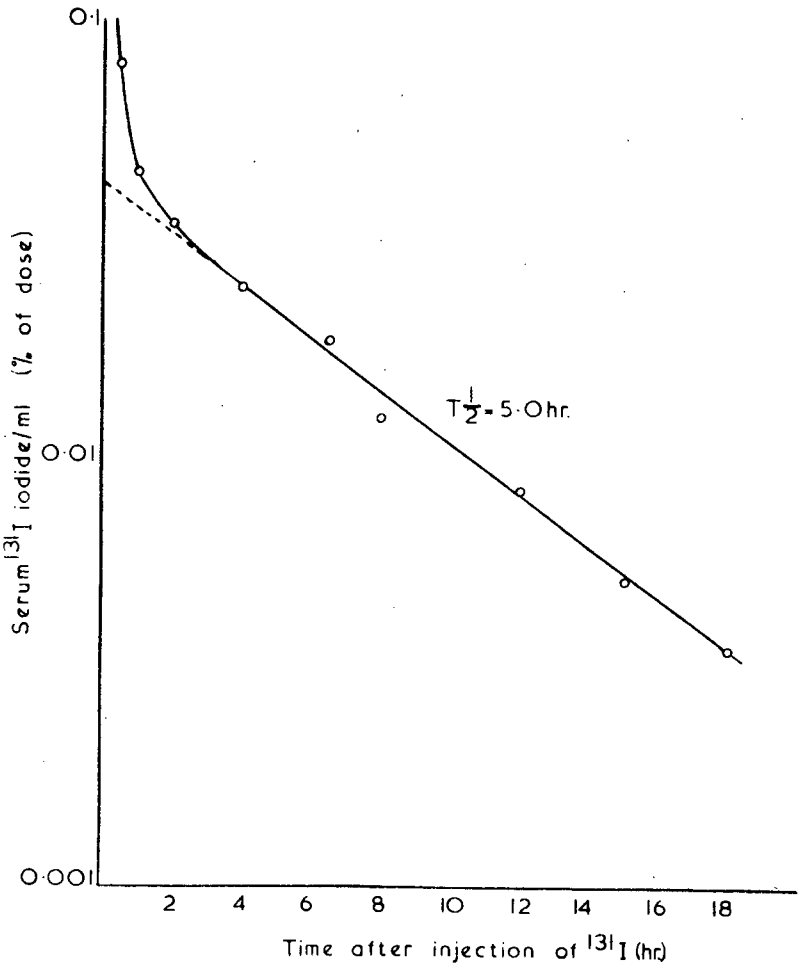

Fig. 9. Variation in the concentration of ^{131}I iodide in the serum of rats during the period 30 min. 9 18 hr. after the injection of ^{131}I.

TABLE 10.

Concentration of ^{131}I in the urine and faeces of rats fed a diet adequate in iodine (30 µg.^{127}I/100g.) and killed 0.5 - 216 hr. after injection of ^{131}I.

Time after ^{131}I hr.	Renal ^{131}I Excretion/hr.*		Faecal ^{131}I Excretion/hr.*	
	µc.	% dose	µc.	% dose
0.5	-	-	-	-
1	-	-	-	-
2	3.210	9.332	-	-
4	2.006	5.814	-	-
6	1.7102	4.971	0.0095	0.028
8	1.4250	4.145	-	-
12	0.7889	2.294	0.0312	0.091
15	-	-	-	-
18	0.3780	1.101	-	-
24	0.2510	0.73	0.0135	0.039
36	0.0965	0.281	0.0395	0.115
48	0.0895	0.260	0.0154	0.045
72	0.0275	0.080	0.0098	0.028
96	0.0240	0.070	0.0139	0.040
120	0.0193	0.056	0.0145	0.042
144	0.0169	0.049	0.0115	0.033
168	0.0141	0.041	0.0128	0.037
192	0.0131	0.038	-	-
216	0.0110	0.032	-	-

* Each value represents the mean of 10 rats.

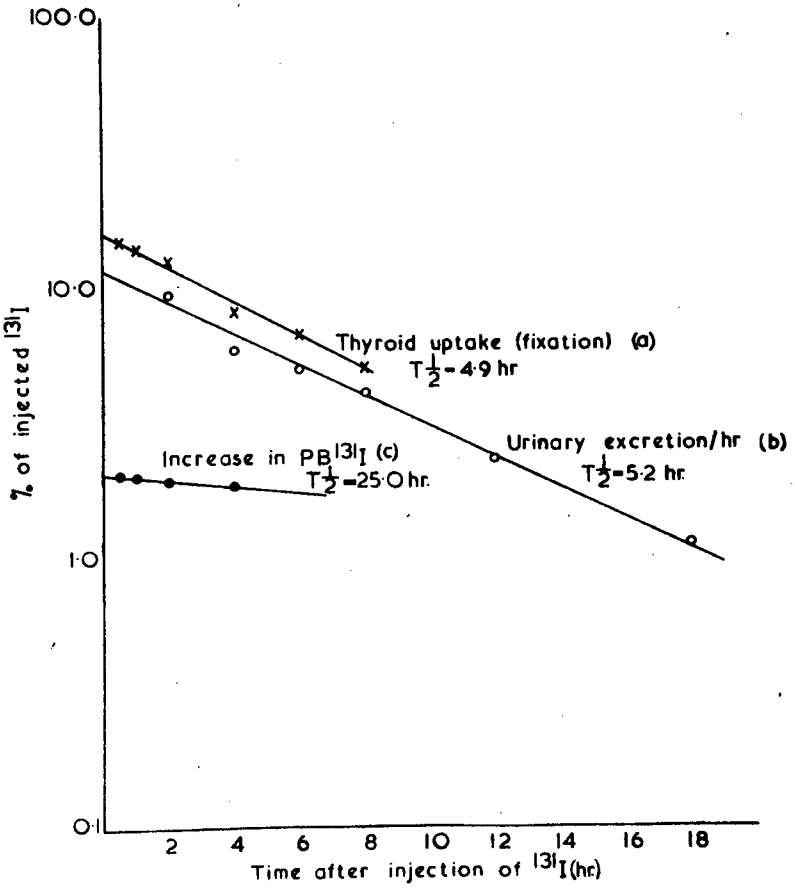

Fig. 10.

Curve (a): Rate of fixation of ^{131}I by the thyroid glands of rats during the period 30 min. - 8 hr. after the injection of ^{131}I.

Curve (b): The renal excretion of ^{131}I in rats during the period 30 min. - 18 hr. after the injection of ^{131}I.

Curve (c): Increase in the concentration of PB^{131}I in the serum of rats during the period 30 min. - 4 hr. after injection of ^{131}I.

The variation in the uptake of the thyroidal ^{131}I during the first 8 hr. was used to construct the curve in Fig.10(a) whereas the renal concentration of ^{131}I during the first 18 hr. was used to obtain Fig.10(b). The values of $(k_T + k_E)$ obtained from the excretion and uptake curves were 0.133/hr. and 0.141/hr. respectively. The constants k_E and k_T can be calculated separately from the uptake of ^{131}I by the thyroid. The thyroidal uptake of ^{131}I is dependent on the partition of the ^{131}I between the thyroid and the kidneys so that the fraction of ^{131}I entering the gland is proportional to the quantity $\frac{k_T}{k_T + k_E}$. It can be seen from equation (4) that at the peak uptake (U max.),

$$U \text{ max.} = \frac{k_T}{k_T + k_E}$$

Consequently, knowing the maximum thyroidal uptake and the constant $(k_T + k_E)$ it is possible to determine the values of constants k_T and k_E. The values of k_T and k_E determined from the measured U max. of 16.5% were 0.023/hr. and 0.115/hr. respectively.

Alternatively according to Brownell (1951), the constants k_T and k_E may be calculated from the concentrations of ^{131}I iodide in the urine and serum. Dividing the concentrations of ^{131}I iodide in the urine at any time t by

the concentration in the plasma at that time, the quantity $k_E (V_2 + V_3)$ can be determined. If the value of $(V_2 + V_3)$ is known, it is possible to find the constants k_E and k_T.

Mean values of 0.026/hr. and 0.112/hr. were calculated for k_T and k_E from the serum and urine data for the period 2 - 18 hr. (TABLES 9 & 10.).

(b) Thyroidal uptake of ^{131}I.

Using the values of k_T and the iodide space derived (see above under Practical Applications of the Model, section (a)) and knowing the concentration of ^{127}I iodide in the iodide space, the rate of trapping of ^{127}I by the thyroid can be calculated provided it is assumed that the uptake of iodide is proportional to the amount of iodide in the iodide space. A circulating ^{127}I iodide concentration of 1.2 µg./100 ml. was measured and therefore the total ^{137}I iodide in the iodide space was 2.92 µg. From these data and using values of k_T of 0.023/hr. and 0.026/hr., the net daily rate of trapping of ^{127}I by the thyroid was calculated to be 1.61 µg. and 1.82 µg. respectively. The thyroidal uptake of ^{127}I iodide was also estimated from the thyroidal ^{131}I clearance rate and the concentration of the circulating ^{131}I and ^{127}I iodide. Clearance values of blood ^{131}I by the rat thyroid were measured during the period 30-120 min. after injection of

^{131}I. A mean value of 6.0 ml./hr. was found. Using these data and a circulating ^{127}I iodide concentration of 1.2 µg./100 ml., the ^{127}I iodide trapped by the thyroid was estimated as 1.73 µg./day.

(c) Thyroid secretion and reaccumulation of ^{131}I released.

An index of the secretion of thyroid hormone can be obtained by estimating the rate of loss of ^{131}I from the thyroid after the maximum uptake. It has been shown by Wolff (1951), Albert (1951) and Perry (1951) that the ^{131}I content of the rat thyroid decreases exponentially with time and when plotted semi-logarithmically, the slope of the straight line represents the ratio of the amount of ^{131}I released per unit time to the total amount in the gland.

In the present study it was assumed that the ^{131}I released from the gland is in the form of T_3 and T_4, and therefore the decrease in thyroidal ^{131}I may be considered to reflect secretion of ^{131}I-labelled T_3 and T_4.

The rate of loss of ^{131}I from the thyroid (k_s) was estimated as 13.1%/day from the slope of the release curve illustrated in Fig. 11 . As already pointed out, (see above under Theoretical Considerations, section (d)) the rate constant (k_s) can also be obtained from the loss

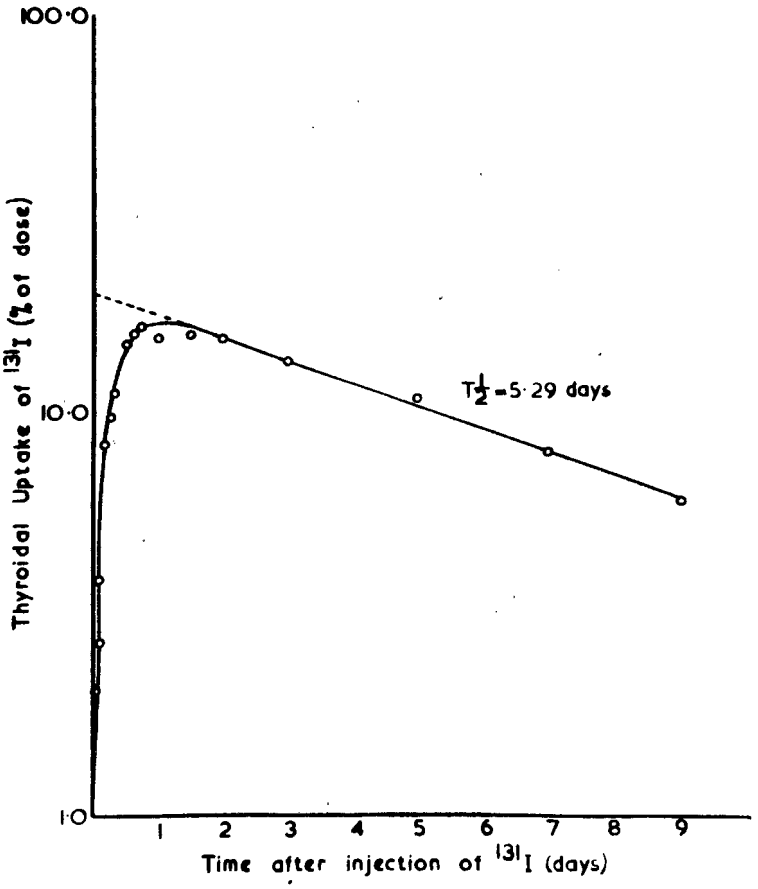

Fig.11. Accumulation and release of thyroidal ^{131}I in rats during the period 30 min. - 216 hr. after injection of ^{131}I.

of ^{131}I from the serum organic iodine pool. The rate constant k_s measured from the variation in the concentration of the $PB^{131}I$ (Fig. 12.) was 0.132/day.

Measurement of the hormonal secretion rate (k_s) from the release curve of unblocked glands is incorrect to the extent that measurement of the thyroidal ^{131}I, at any time following the peak uptake, will represent a small fraction of ^{131}I that has been secreted and reaccumulated by the thyroid. Similarly, measurement of k_s from the $PB^{131}I$ will also be incorrect to the same extent.

In order to determine the extent of this re-accumulation, the partition of ^{131}I between the thyroid and the kidneys was calculated from the urinary and thyroidal content of ^{131}I for the rats killed 2 hr. and 4 hr. after the administration of ^{131}I. Expressing the fraction of the ^{131}I in the thyroid as a percentage of that excreted in the urine (the partition factor) a mean value of 19.17% was obtained. The mean daily renal excretion of ^{131}I and the mean daily loss of ^{131}I from the thyroid were determined from the data in TABLES 9 & 10. for the interval 48-216 hr. and found to be 0.431 µc. and 0.471 µc. respectively. The product of the partition factor and the mean daily renal excretion of ^{131}I iodide during the release curve will give a measure of the ^{131}I reaccumulated by the thyroid. The fraction of the ^{131}I

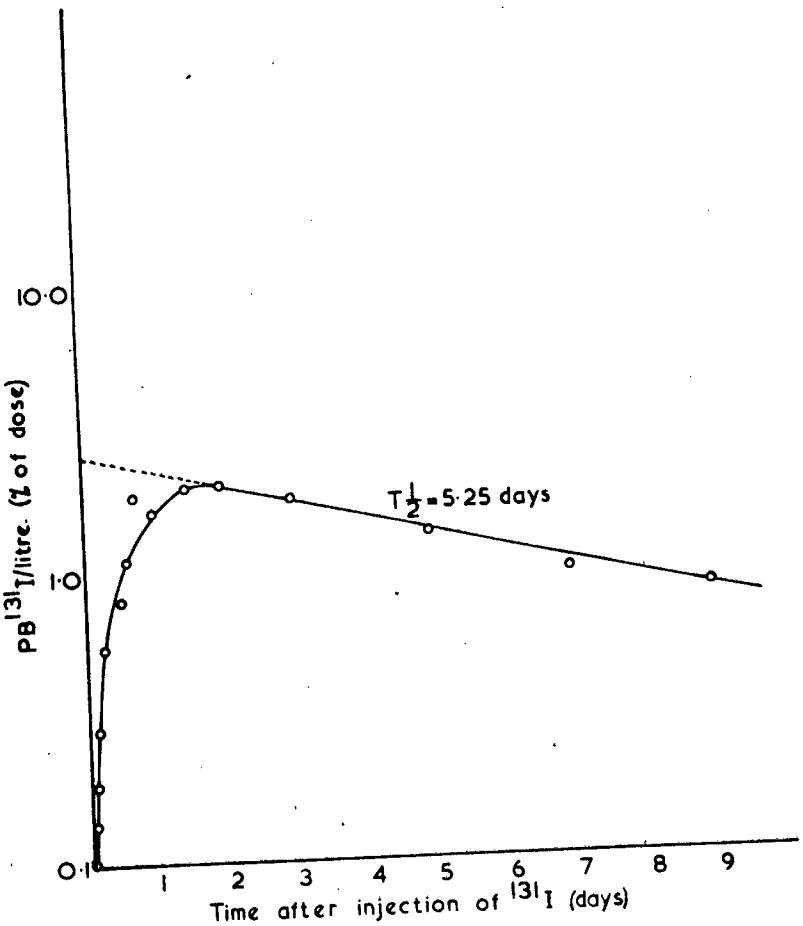

Fig. 12. Variation in the concentration of $PB^{131}I$ in the serum of rats during the period 30 min. - 216 hr. after injection of ^{131}I.

reaccumulated per day calculated in this way was found to be 0.0826 μc. Therefore, since the measured loss of ^{131}I from the thyroid was 0.471 μc./day, the actual loss would then be 0.5536 μc./day. Consequently, each day 14.9% of the ^{131}I secreted as hormone is reutilized by the thyroid.

It should be possible to calculate the fraction of ^{131}I reaccumulated by the thyroid (see above under Theoretical Considerations, section (b)) by subtracting from the measured amount of ^{131}I in the gland at any time after 48 hr., the quantity of ^{131}I calculated from equation (5). The measured uptakes and the calculated uptakes are compared in TABLE 11. The mean difference which corresponds to the fraction of ^{131}I utilized by the thyroid is 14.4%.

It may be seen from equation (9) that the daily reutilization of ^{131}I by the thyroid can be derived from the measured thyroidal loss of ^{131}I and the rate of excretion of ^{131}I in the urine (k'_E) during the period 72-216 hr., when the fraction of unmetabolised ^{131}I iodide remaining from the initial dose is assumed to be negligible. The variation in serum ^{131}I iodide (Fig. 13) during the period 72-216 hr. indicates that the concentration of ^{131}I iodide is extremely low, thus supporting the latter consumption. Substituting the value of k_s previously measured from the release curve 0.131/day and the value of 0.113/day for k'_E (derived from the slope of latter portion of the excretion curve,

TABLE 11.

Determination of the fraction of ^{131}I secreted as hormone reutilized by the thyroid gland by subtracting the thyroidal uptake of ^{131}I calculated by equation 5 (see under Theoretical Considerations, section b) from the measured uptake.

Time after ^{131}I hr.	Thyroid ^{131}I uptake		Amount ^{131}I reutilized μc.	Fraction ^{131}I reutilized(F) * %
	Measured μc.	Calculated μc.		
48	5.36	4.68	0.68	14.5
72	4.65	4.10	0.55	13.4
120	3.72	3.08	0.64	17.9
168	2.73	2.40	0.33	13.8
216	2.06	1.83	0.23	12.6

* Percentage of calculated thryoidal uptake of ^{131}I.

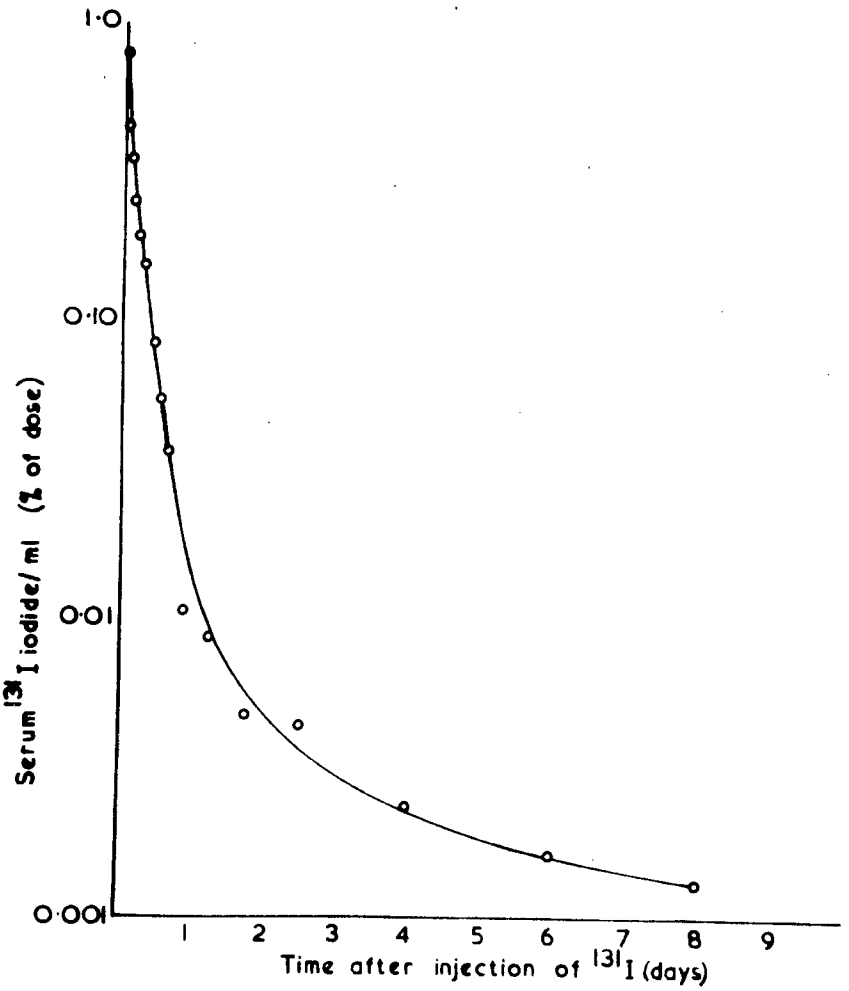

Fig.13. Variation in the concentration of ^{131}I iodide in the serum of rats during the period 30 min. - 216 hr. after injection of ^{131}I.

Fig. 14.) in equation (9), the fraction of the secreted ^{131}I reaccumulated by the thyroid was calculated as 14.4%/day.

The fraction of reaccumulated ^{131}I was also estimated from the thyroidal ^{131}I clearance rate and the concentration of serum ^{131}I iodide at later intervals after injection of ^{131}I, when it is assumed that all the ^{131}I iodide appearing in the blood is derived from the degradation of hormone in the peripheral tissues. Using the data that the mean ^{131}I iodide concentration of the serum for the period 72-216 hr. was 4.7×10^{-4} µc./ml. and that the thyroidal clearance rate of ^{131}I was 6.0 ml./hr., it was calculated that the ^{131}I iodide was trapped at a rate of 0.0676 µc./day during this period. This corresponds to a daily reaccumulation of 14.4% of the ^{131}I released from the thyroid as labelled hormone.

At later time intervals, in the event of the excretion of organic bound iodine being zero (i.e. $k_F = 0$), the fraction of ^{131}I reaccumulated by the thyroid would be equal to $\frac{k_T}{k_T + k_E}$. In other words the fraction reaccumulated would be equal to the maximum uptake of ^{131}I by the thyroid. In the present study the maximum uptake was 16.5% whereas the mean value for the fraction reaccumulated was 14.6%.

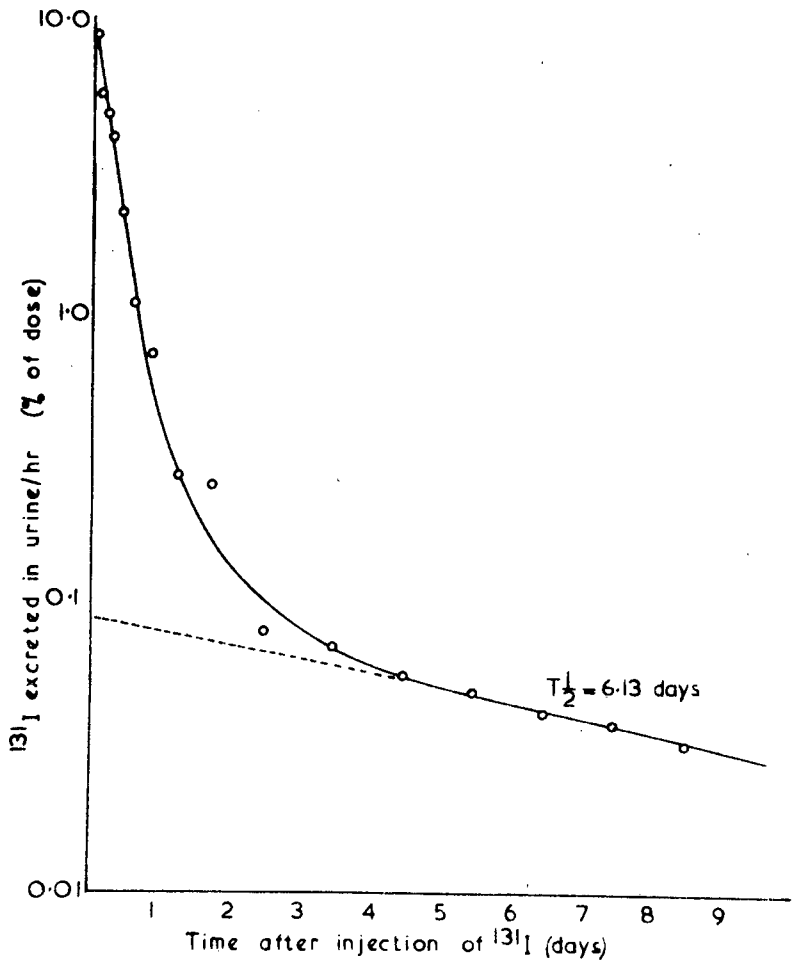

Fig.14.The renal excretion of ^{131}I in rats during the period 30 min. - 216 hr. after the injection of ^{131}I.

Knowing the fraction of ^{131}I reutilized by the thyroid, the actual rate of secretion of labelled hormone (k_s') was calculated from equation (7) as 0.152/day. A mean value of 14.6% for the fraction of ^{131}I reutilized was used for this determination. Since the mean total iodine content of the gland is 11.55 μg. (Chapter 3, Part II), the absolute rate of secretion of hormone from the gland calculated from k'_s was 1.76 μg.^{127}I/day which is equivalent to 2.69 μg.T_4/day.

Assuming the rate of secretion of T_3 and T_4 to be proportional to the concentration of T_3 and T_4 in the gland, the absolute rate of hormone secretion can be measured from the rate of increase of ^{131}I in the PBI pool (k_o) during the first few hours after injection of ^{131}I. In order for this to be true the degradation and the excretion of organic bound ^{131}I must be negligible during the early intervals. From the data in TABLE 9, the rate of increase of the PB^{131}I for the period 0.5-4hr. was found to be 6.65%/day (Fig. 10(c)). In this study the concentration of stable iodine in T_3 and T_4 was not measured but using the values of T_4 (2.040 μg.) and T_3 (0.263 μg.) measured in Part II (a) of the investigation of rats on normal iodine diet (Chapter 3.), the absolute rate of hormone secretion was calculated to be 1.53 μg.^{127}I/day which is equivalent to 2.34 μg.T_4/day.

If the thyroid gland is in a steady state, the absolute rate of glandular trapping of iodine should equal the absolute rate of release of hormonal iodine from the thyroid. Therefore, since the mean trapping rate of iodine was calculated at 1.72 µg./day the hormonal secretion rate should on this basis be 1.72 µg.^{127}I/day. This is equivalent to 2.64 µg.T_4/day.

(d) Metabolic degradation of hormonal iodine.

When isotopic equilibrium exists, the rate of metabloic degradation of organic iodine (k_D) can be measured by the sum of the rate constants k_T and k_E. Since the mean value of the constant ($k_T + k_E$) was found to be 0.138/hr. and the concentration of the PB^{127}I was 3.5 µg./100 ml., it could be calculated that 1.82 µg.^{127}I/day was released by degradation of organic iodine. This is equivalent to 2.78 µg./T_4/day.

If a steady state exists in the animal, the absolute rate of release of ^{127}I from the thyroid will equal the absolute rate of degradation. Consequently, in the present study 1.82 µg.^{127}I was secreted per day as hormone which is equivalent to 2.78 µg.T_4/day. In addition, the absolute glandular trapping rate of iodine should equal the absolute rate of release from the thyroid if the animal is in a steady state. Therefore, since the mean glandular trapping rate of iodine was calculated at 1.72 µg./day,

the degradation rate is equivalent to 2.64 $\mu g.T_4$/day.

The thyroxine distribution space (TDS) can be obtained by extrapolation of the semi-logarithmic plot of the rate of decrease in $PB^{131}I$ to zero time. Using the value of 26.9 ml. for the TDS derived from Fig. 12 and a concentration of 3.5 $\mu g.^{127}I$/100 ml. for the PBI, the turnover time of the extrathyroidal organic iodine was calculated from the degradation rate to be 0.76 days.

DISCUSSION.

It may often be possible to reduce the number of compartments of a model system so that the basic elements of the problem under consideration are still retained. For example, if the interactions between certain compartments occur rapidly it may be possible to consider them as a single compartment. In the model used in the present study, the diffusion of ^{131}I iodide from the plasma into the tissue fluids was considered to be complete within 2 hr. so that after this time the plasma and tissue fluid volumes could be treated as a single volume. Oddie, Meschan and Wortham (1955) feel that the equations developed by treating the iodide space as a single volume only account approximately for the behaviour of the injected ^{131}I in the later metabolic stages. However, in the present investigation wherever it has been possible to compare the

experimentally determined results with those derived from the equations, the values have agreed satisfactorily.

Evidence in favour of the diffusion into the tissue fluids being essentially complete within 2 hr. was obtained from the semi-logarithmic plot of the variation in serum ^{131}I iodide concentration with time (Fig. 9.). It may be seen that the curve is made up of two exponential terms. The first component with a faster rate constant and which predominates until approximately 2 hr. represents the diffusion of the ^{131}I iodide from the blood into the tissue fluids. The second slower component represents the removal of ^{131}I iodide from the blood by the thyroid and the kidneys. The constant $(k_T + k_E)$ was measured from the slope of the second component for the period 2-18 hr. after injection of ^{131}I during which time the contribution made by the slower compartments was regarded as negligible. Good evidence for this assumption is the fact that the volume of the iodide space, obtained by extrapolation of the second exponential to zero time, is very nearly equal to the total body water. Determined on the basis of desiccation studies (Annegers, 1954) the total body water for a rat of average weight of 300 g. would be 220 ml. The volume of the iodide space obtained from the serum iodide curve is 244 ml. which is in close agreement indicating that the assumptions made about the iodide pool

sizes in the early stages are probably valid.

The thyroid gland has been considered to act as a single iodine compartment although it is really a highly complex multi-compartment system. This assumption has been made on the basis that the rate of release of labelled iodine from the thyroid is a simple exponential function (Wolff, 1951; Albert, 1951; Perry, 1951).

It might be expected from the model that the ^{131}I release curve would show an initial rapid phase of disappearance as the labelled hormone leaves the gland. However, when sufficient ^{131}I iodine has become available from peripheral degradation of hormone for reutilization by the thyroid, there would be stabilization of the release curve at a slower rate.

In the present investigation the loss of ^{131}I from the gland was only slightly more rapid in the earlier than the latter stages. The biphasic nature of the decay curve is not as striking as might be expected probably for two reasons. (1) the iodine store in the gland was large (2) the duration of study was too short. It might be expected on these grounds that a biphasic release curve would exist in thyroids with low iodine stores, having a faster turnover. This has in fact been demonstrated in another study on rats fed a low iodine diet (Chapter 4.).

The simple exponential rate of release of ^{131}I from the glands of normal rats and the biphasic nature of the release curves observed in glands of rats fed a low iodine diet, provide strong evidence that the iodine within the gland acts as a single compartment.

It has been assumed that the ^{131}I released from the gland is entirely in the form of T_3 and T_4. However, in addition to the loss of ^{131}I from the thyroid as labelled hormone, ^{131}I could be lost as inorganic ^{131}I. This could occur as a result of the exchange of circulating ^{127}I iodide with ^{131}I present in the exchangeable thyroidal inorganic iodide pool, but since at least 99% of the total thyroidal iodine is organically bound this factor would be of relatively minor importance. Loss of ^{131}I iodide could also arise as the result of intra-thyroidal deiodination of labelled MIT and DIT. Losses of ^{131}I due to deiodination may well be extensive depending on the extent of intra-thyroidal recycling mechanism. It is possible that measurements of absolute hormone secretion rates from ^{131}I release curves, even after correction has been made for the re-utilization of ^{131}I released as hormone, are incorrect due to losses of ^{131}I from the gland by deiodination.

This is borne out by the fact that the rate of secretion of hormonal iodine calculated from the concentration

of the stable iodine in T_3 and T_4 in the gland was considerably lower than the values calculated on the basis of iodide trapping and release, or metabolic degradation. The actual difference was 0.24 µg. ^{127}I/day which indicates that this amount of iodine is lost each day from the thyroid gland as iodide.

In addition, it is possible that organically bound ^{131}I may be lost from the thyroid in forms other than T_3 and T_4. Small amounts of labelled MIT and DIT have been noted in the serum of normal humans (Benua and Dobyns, 1955) and in the venous blood of the thyroids of dogs (Matsuda, Shimoda and Green, 1964). In isotope equilibrium studies Pitt-Rivers and Rall (1961) showed that in rats the ^{131}I in the combined MIT and DIT fraction amounted to 1 - 3% of the total serum ^{131}I. Also analysis by gel filtration of labelled iodinated constituents in the serum of rats isotopically equilibrated with ^{125}I did not reveal appreciable quantities of MIT or DIT (Lissitzky, Bismuth and Simon, 1963). It therefore seems likely that the loss of organic iodine from the thyroid is almost entirely in the form of T_3 and T_4.

The estimations of the rates of hormonal secretion and degradation obtained in the present study agree with those obtained by Gregerman (1963) using the radioactive thyroxine turnover technique.

Part II : INTRATHYROIDAL IODINE METABOLISM

METHODS

(For detailed descriptions of the procedures used refer to Chapter 1.)

EXPERIMENT 1 : Rats killed at intervals 2 - 168 hr. after injection of ^{131}I.

Male Sprague-Dawley rats weighing 317 $\pm$ 12g. were used. The animals were fed a commercial pellet diet (30 µg. iodide/100g.) and had free access to tap water.

Approximately 50 µc. carrier-free $Na^{131}I$ was injected intraperitoneally into each rat and thereafter the animals were killed at intervals ranging from 2 - 168 hr. At each time interval six rats were killed, their thyroid glands were removed and counted for uptake of injected ^{131}I. The glands were hydrolysed enzymically and labelled iodoamino acids in each hydrolysate were separated by paper chromatography in BDA and BAW. The distribution of ^{131}I and ^{127}I between the various iodoamino acids was measured and their absolute specific activities determined. The total thyroidal iodine content was determined by analysis of 20 µl. portions of the thyroid hydrolysates of rats killed at 120 hr.

and 168 hr. At certain intervals the distribution of ^{131}I and ^{127}I iodide in thyroid homogenates and hydrolysates was measured using both paper chromatography (BAW) and electrophoresis on cellulose acetate strips. In these instances the thyroids were immediately homogenized in 80 µl. thiouracil in ice and 25 µl. portions of the homogenate taken for electrophoresis and paper chromatography.

EXPERIMENT 2 : Rats killed at intervals 30 sec. - 90 min. after injection of ^{131}I.

Male Sprague-Dawley rats weighing 304 $\pm$ 10g. were used. The animals were maintained on the same diet as those used in Experiment 1.

Approximately 200 µc. carrier-free $Na^{131}I$ was injected intravenously into each rat and the animals were killed at intervals ranging from 30 sec. to 90 min. after injection. When the time between injection and autopsy was 5 min. or less, the rats were kept under ether anaesthesia for the entire period with their thyroids partly exposed but still intact. The glands were exposed by making a mid-line incision on the ventral surface of the skin of the neck. The sterno-thyroid muscle was subsequently divided in the mid-ventral position. The two

parts of the muscle sheath were drawn laterally to expose the thyroid lobes for rapid removal. At this stage the ^{131}I was injected intravenously. At each interval six rats were killed, the thyroids were immediately removed and plunged into boiling thiouracil, homogenized and boiled for 3 min. The boiled homogenates were enzymically hydrolysed.

The distribution of ^{131}I between the various iodoamino acids in the thyroid hydrolysates was measured and their absolute specific activities were determined.

RESULTS

EXPERIMENT 1 : Rats killed at intervals 2 - 168 hr. after injection of ^{131}I.

Uptake of ^{131}I.

The uptake of ^{131}I by the thyroid reached a maxium of 17.3% of the injected dose at 18 hr. and fell to 8.3% after 168 hr. The release of ^{131}I from the thyroid is shown in Fig. 15 . The biological half-life ($T_{\frac{1}{2}}$) of the thyroidal ^{131}I, as determined from the slope of the release curve, was 5.25 days.

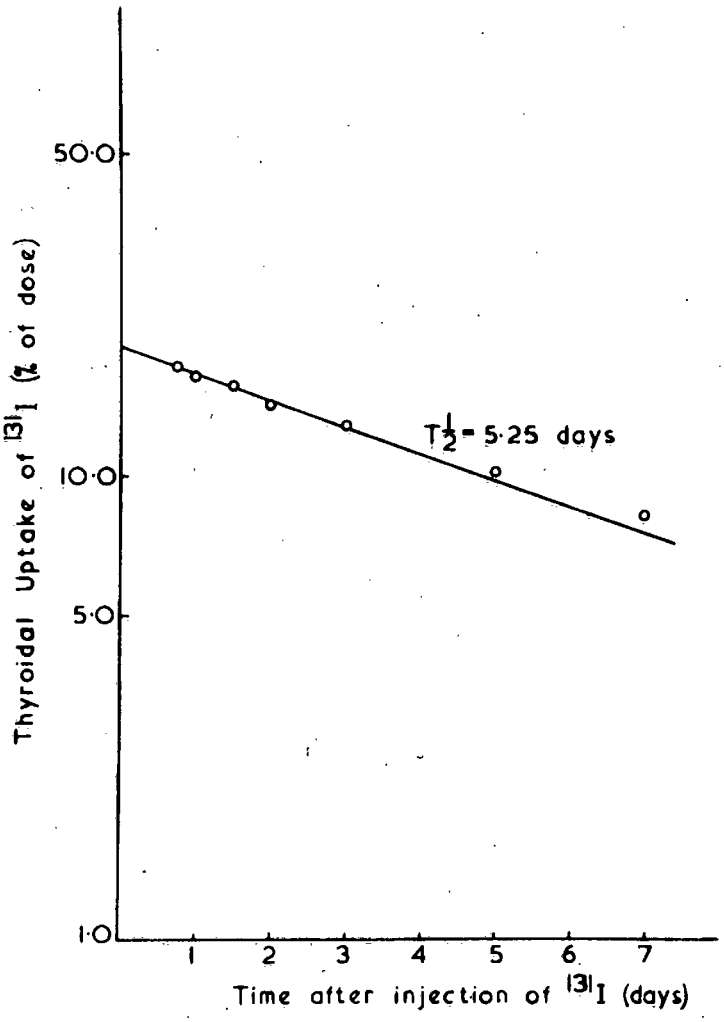

Fig. 15. The release of ^{131}I from the thyroid glands of rats on a diet adequate in iodine (30 µg.^{127}I/100g.)

Distribution of ^{131}I in the thyroid glands.

a) Unhydrolysed glands.

The distribution of ^{131}I in unhydrolysed thyroid homogenates of rats killed 2, 6, 48 and 72 hr. after injection of ^{131}I was determined both by electrophoresis and paper chromatography. At all time intervals investigated the thyroglobulin and the inorganic iodide accounted for practically all of the ^{131}I. Trace amounts of free labelled iodotyrosines were noted only at the later intervals in the chromatographed homogenates.

It may be seen from the data in TABLE 12. that at all intervals investigated the percentage of inorganic thyroidal ^{131}I in the homogenates was slightly greater when determined by paper chromatography. When determined by electrophoresis, the inorganic thyroidal ^{131}I in the homogenates accounted for 2 - 4% of the total thyroidal ^{131}I at early intervals, whereas at 48 hr. and 72 hr. the inorganic ^{131}I fraction represented approximately 0.30% of the total ^{131}I.

b) Hydrolysed glands.

The enzymic hydrolysis of thyroglobulin was performed quantitatively and the nonhydrolysed material represented only about 5% of the total radioactivity on the chromatogram.

TABLE 12.

Determination of the fraction of total thyroidal ^{131}I present as inorganic iodide in thyroidal homogenates and hydrolysates of rats using electrophoresis and paper chromatography. Rats on a normal iodine diet (30 µg.^{127}I/100g) killed at intervals 2 - 72 hr. after injection of ^{131}I.

Interval after ^{131}I hr.	ELECTROPHORESIS *		CHROMATOGRAPHY*		
	homogenate %	hydrolysate %	homogenate BAW %	hydrolysate BAW %	hydrolysate BDA %
2	4.03	7.07	4.48	8.22	8.07
4	3.06	6.89	3.63	7.14	7.86
6	1.91	5.49	2.20	6.38	6.92
48	0.34	4.20	0.72	4.92	5.50
72	0.28	4.37	0.88	5.06	5.23

* Each value represents the mean of six analyses.

The distribution of ^{131}I between the various iodoamino acids in thyroid hydrolysates for the period 2 - 168 hr. after administration of ^{131}I is shown in Fig. 16. The data used for plotting the curves illustrated in Fig.16. are presented in TABLE 13. It is seen that the percentage of the total thyroidal ^{131}I present as iodotyrosines always exceeded that of the iodothyronines. MIT and DIT were labelled much more rapidly than T_3 and T_4 and had already reached maxium labelling 2 hr. after injection, whereas the relative abundances of T_3 and T_4 became maximal only after 36 - 48 hr. The equilibrium percentage distribution of ^{131}I in MIT, DIT, T_4 and T_3 was 22.6, 42.4, 17.4 and 2.1 respectively.

Following the initial rapid rise, the percentage of ^{131}I in MIT and DIT diminished significantly during the period 4 - 24 hr. and thereafter approached the apparent equilibrium values. The decrease in the percentage label in MIT was much greater than that of DIT. At all times the percentage of the thyroidal label in DIT exceeded that of MIT so that the ratio of labelled MIT to labelled DIT (R value) was always below unity (TABLE 13). The R values did not change significantly during the time elapsing after injection as would be expected if MIT were the immediate precursor of DIT. There was however, a gradual decrease in the R value from 0.70 at early

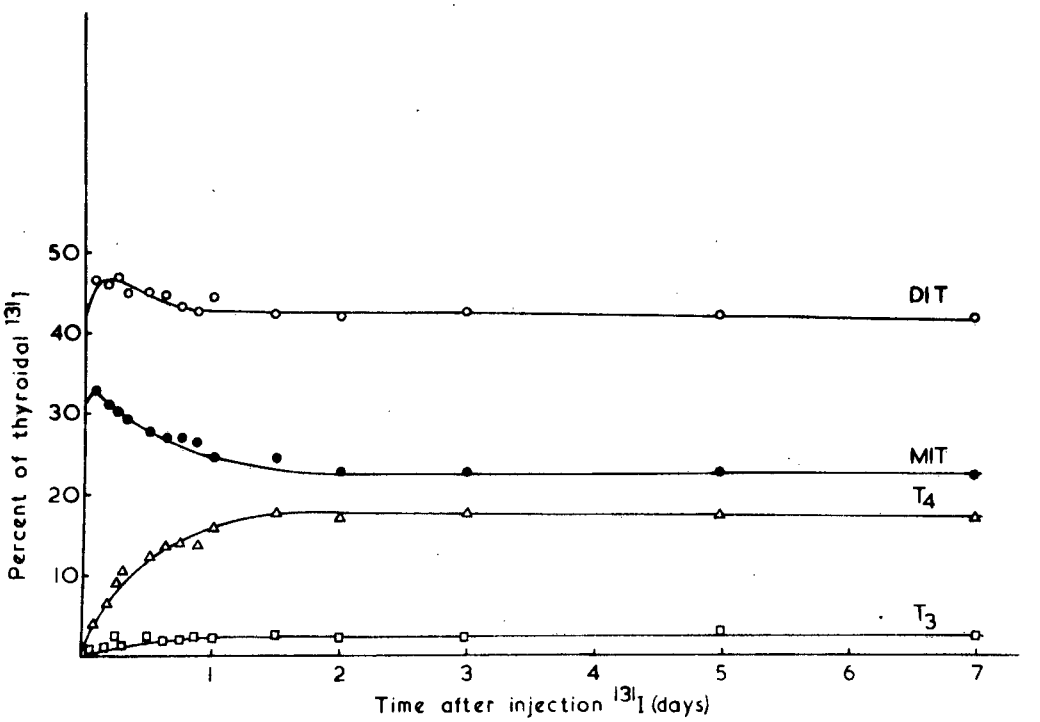

Fig. 16. Distribution of ^{131}I between the various iodoamino acids in thyroid hydrolysates of rats on a diet adequate in iodine (30 µg.^{127}I/100g.), killed 2 - 168 hr. after injection of ^{131}I.

TABLE 13.

The distribution of ^{131}I between the components of thyroid hydrolysates of rats on a normal iodine diet (30 µg.^{127}I/100g.), killed 2 - 168 hr. after injection of ^{131}I.

Time after injection hr.	% distribution of ^{131}I *					MIT/DIT	T_3/T_4
	I	MIT	DIT	T_4	T_3		
2	8.22	32.80	46.55	3.94	0.62	0.71	0.16
4	7.14	31.06	46.10	6.25	0.87	0.67	0.14
6	6.38	30.14	46.89	9.37	1.21	0.67	0.13
8	4.30	29.09	44.90	10.22	1.08	0.65	0.11
12	3.41	27.87	45.08	12.20	2.03	0.62	0.17
15	5.05	27.03	44.71	11.90	1.71	0.60	0.14
18	5.27	26.90	43.35	13.97	1.80	0.62	0.13
21	3.97	26.64	42.92	13.72	2.06	0.62	0.15
24	5.10	24.33	44.33	15.90	1.91	0.55	0.12
36	4.01	24.38	42.59	17.71	2.58	0.57	0.15
48	4.92	22.60	42.33	17.28	2.17	0.53	0.13
72	5.06	22.89	42.81	17.81	2.02	0.49	0.12
120	5.62	22.58	42.50	17.22	2.22	0.50	0.14
168	5.70	22.37	42.01	17.30	2.14	0.50	0.13

* Each value represents the mean 6 rats.

intervals to 0.50 at 168 hr.

Both ^{131}I labelled T_3 and T_4 increased gradually with time reaching a maxium at about 36 hr. Their increase corresponded with the relative diminution in labelled MIT and DIT during the period 4 - 24 hr. The percentage of radioactivity in T_4 at all times exceeded that in T_3. The ratios of labelled T_3 to labelled T_4 did not show any significant change with time (TABLE 13).

At all intervals the inorganic ^{131}I resolved from the thyroid hydrolysates by paper chromatography was in excess of that determined by electrophoresis of homogenates and remained constant at 4 - 5% of the total thyroidal ^{131}I except at 2 - 6 hr. when it represented approximately 7%.

The inorganic ^{131}I in the thyroid hydrolysates of rats killed at 2, 4, 6, 48 and 72 hr. was determined by electrophoresis in order to show whether the excess iodide found after chromatography of the hydrolysates was produced during hydrolysis or paper chromatography. TABLE 12 compares the percentage of the total thyroidal ^{131}I present as inorganic ^{131}I when measured after either electrophoresis or paper chromatography of thyroid homogenates or hydrolysates. TABLE 12. also shows that the percentage of ^{131}I determined from chromatograms developed

in BDA was slightly higher than that determined from chromatograms developed in BAW.

Distribution of ^{127}I in the thyroid glands.

a) Unhydrolysed glands.

The total iodide content of the thyroid gland was determined by direct stable iodine analysis of the ^{131}I iodide fractions separated by electrophoresis. Analyses of 24 glands gave a value of 0.033 $\pm$ 0.005 µg. iodide (mean $\pm$ S.D.) which is equivalent to 0.29% of the total iodine of the gland (11.55 µg.)

b) Hydrolysed glands.

Quantitative (µg./gland) and relative (% gland) distributions of the thyroidal iodoamino acids and of their iodine content were measured in the hydrolysates of the rats killed after 120 and 168 hr. (TABLE 14). The recoveries of ^{127}I after paper chromatography were quantitative. The mean recovery of ^{127}I based on the total iodine content of unchromatographed hydrolysates was 90%.

Since the origin material never accounted for more than 6% of the total ^{127}I the molar ratios of iodoamino acids could be calculated from the distribution of the ^{127}I. The molar ratios of MIT : DIT : T_4 : T_3 were

TABLE 14.

The distribution of ^{127}I and of iodine containing compounds in the thyroid glands of rats on a diet containing 30 µg.^{127}I/100g.

	Stable iodine content/gland*		Iodoamino acid content/gland *	
	µg.	%	µg.	%
Total I	11.547	100.0	-	-
MIT	2.595	22.4	6.27	33.0
DIT	5.011	43.4	8.53	44.9
T4	2.040	17.7	3.12	16.4
T3	0.263	2.3	0.49	2.6
Iodide (electrophoresis)	0.033**	0.3	0.03 **	0.2
Iodide (chromatography)	0.586	5.1	0.59	3.1
origin	0.671	5.8	-	-
other	0.381	3.3	-	-
MIT/DIT	0.52		0.73	
T3/T4	0.13		0.16	

* Each value represents the mean of 12 rats

** Determination of the thyroidal iodide content using electrophoresis was performed on 24 rats.

27 : 26 : 5: 1.

Comparing the distribution of the ^{127}I with that of the ^{131}I in the rats killed at 120 hr. and 168 hr. after ^{131}I injection it may be seen that the newly acquired ^{131}I in all the iodoamino acids has an equilibrium distribution almost identical with that of the pre-existing ^{127}I. Assuming the same ^{127}I distribution in all thyroid glands analysed in the present experiment then this equilibrium was essentially complete within 36 hr.

Absolute specific activity of the iodocompounds.

The incorporation of ^{131}I into the various thyroidal iodoamino acids has been compared with the values of ^{127}I in each iodoamino acid. The results were expressed in terms of absolute specific activity of the iodine in the particular iodoamino acid ($\mu c.^{131}I/\mu g.^{127}I$).

a) Inorganic iodide.

The specific activities of the inorganic iodide of unhydrolysed thyroid homogenats of the rats killed 2, 4, 6, 48 and 72 hr. after injection of ^{131}I were measured using electrophoresis and paper chromatography (TABLE 15). At early intervals the specific activities of iodide obtained by electrophoresis were much higher than those obtained by paper chromatography either before or after hydrolysis of the homogenate. At 48 hr. and

TABLE 15.

Measurement of the specific activity ($\mu c.^{131}I/\mu g.^{127}I$) of thyroidal inorganic iodide at different time intervals after injection of ^{131}I using electrophoresis and chromatography.

Interval after ^{131}I hr.	ELECTROPHORESIS*	CHROMATOGRAPHY*	
	Homogenate $\mu c.^{131}I/\mu g.^{127}I$	Homogenate $\mu c.^{131}I/\mu g.^{127}I$	Hydrolysate $\mu c.^{131}I/\mu g.^{127}I$
2	10.529	7.115	0.550
4	6.648	4.663	0.638
6	3.750	3.026	0.871
48	0.601	0.710	0.725
72	0.591	0.612	0.603

* Each value represents the mean of six analyses.

72 hr. however, the specific activities of iodide in unhydrolysed glands became similar to those of hydrolysed glands.

b) Iodoamino acids.

The specific activities of the iodine in the various thyroidal iodoamino acids resolved from enzymic hydrolysates by paper chromatography were measured as a function of time. These data are presented in TABLE 16 .

The graphical procedure of Zilversmit, Entenman and Fishler (1943), which is based on the analysis of specific activity - time curves, was used for testing the identity of a simple precursor-product relationship. Such specific - time curves are illustrated in Fig. 17 .

Fig. 17. shows that there was no simple precursor-product relationship between MIT and DIT or T_3 and T_4. The specific activity of MIT was always higher than that of DIT until approximately 3 days when the specific activities of MIT and DIT gradually became equal. The peak specific activity of MIT occurred at 18 - 21 hr. whereas the peak specific activity of DIT was slightly later occurring at 21 hr. At all intervals the specific activity of the iodotyrosines exceeded that of the iodothyronines except after 3 days when specific activities became equal and remained equal. In other

TABLE 16.

Absolute specific activity of iodine in the components of thyroid hydrolysates of rats on a normal diet (30 μg.^{127}I/100g.), killed 2 - 168 hr. after injection of ^{131}I.

Interval after ^{131}I hr.	Specific activity (μc.^{131}I/μg.^{127}I)*				MIT/DIT	T3/T4
	MIT	DIT	T4	T3		
2	0.288	0.219	0.058	0.093	1.31	1.60
4	0.475	0.361	0.144	0.226	1.32	1.57
6	0.554	0.465	0.246	0.318	1.19	1.29
8	0.781	0.550	0.318	0.388	1.42	1.22
12	0.867	0.785	0.560	0.624	1.10	1.11
15	0.930	0.803	0.585	0.681	1.16	1.16
18	0.944	0.835	0.670	0.767	1.13	1.15
21	0.941	0.848	0.710	0.771	1.10	1.09
24	0.910	0.814	0.715	0.782	1.12	1.09
36	0.829	0.750	0.694	0.719	1.10	1.04
48	0.765	0.701	0.630	0.676	1.09	1.07
72	0.609	0.586	0.562	0.570	1.04	1.01
120	0.441	0.430	0.425	0.431	1.03	1.02
168	0.318	0.314	0.312	0.320	1.01	1.03

* Each value represents the mean of 6 rats.

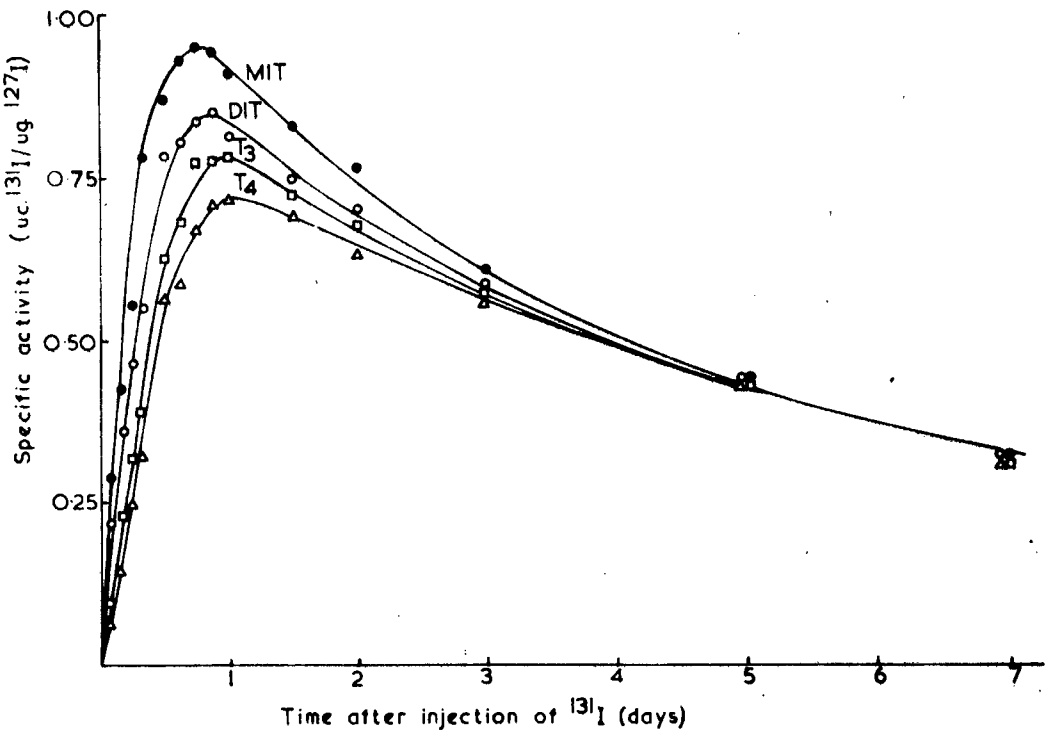

Fig. 17. Variations in absolute specific activity of iodine in the different iodoamino acids of thyroid hydrolysates of rats fed a diet adequate in iodine (30 µg. ^{127}I/100g.). Rats killed at intervals 2 - 168 hr. after injection of ^{131}I.

words, at approximately 3 days the ^{131}I in all the thyroidal iodoamino acids had reached isotopic equilibrium.

The specific activities of T_3 and T_4 increased less rapidly then MIT and DIT and the peak of both iodothyronines occurred simultaneously at 24 hr. which is later than any of the iodotyrosine peaks. At early intervals the specific activity of T_3 increased more rapidly than that of T_4 with the result that specific activity of T_3 was always higher until isotopic equilibrium was reached.

The ratio (S) of the specific activity of MIT to that of DIT was found to decrease gradually from 1.31 to unity during the period of the experiment (TABLE 16). Similarly the ratio of the specific activity of T_3 to T_4 decreased gradually from 1.60 to unity.

EXPERIMENT 2 : Rats killed at intervals 30 sec. - 90 min. after injection of ^{131}I.

Since the results of Experiment 1 showed no precursor-product relationship between MIT and DIT it was of interest to study in detail the early time intervals after the injection of ^{131}I.

Distribution of ^{131}I in hydrolysed glands.

The distribution of ^{131}I in the thyroid hydrolysates of rats killed 30 sec. - 90 min. after intravenous injection of ^{131}I is shown in Fig. 18. The data for Fig. 18. are presented in TABLE 17.

After 30 sec., 21% of the total thyroidal ^{131}I was already protein-bound in the form of MIT and DIT. The proportion of protein-bound ^{131}I increased rapidly and by 10 min. over 70% of the total ^{131}I was in the form of iodotyrosines. The relative abundances of labelled MIT and DIT became maximal within 20 - 40 min. and at all times the radioactivity in DIT was greater than MIT. MIT and DIT were labelled much more quickly than T_3 and T_4. The labelled iodothyronines were first detected approximately 20 min. after injection but the activity was too low for analysis.

Absolute specific activity of the iodine in the iodocompounds.

Fig. 19. indicates that from the earliest times investigated the specific activity of the iodine in MIT was greater than that of DIT. However, it was found that the ratio of the specific activity of MIT to DIT decreased with time (TABLE 18.).

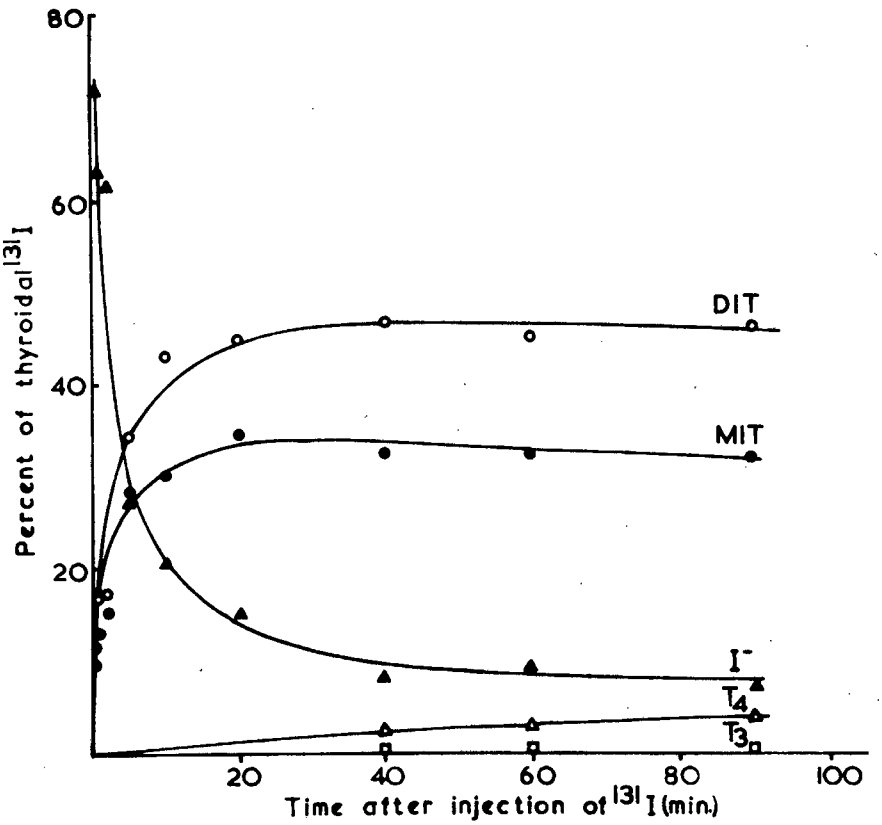

Fig. 18. Distribution of ^{131}I between the various iodoamino acids in thyroid hydrolysates of rats on a diet adequate in iodine (30 µg.^{127}I/100g.), killed 30 sec. - 90 min. after injection of ^{131}I.

TABLE 17.

The distribution of ^{131}I between the components of thyroid hydrolysates of rats on a normal iodine diet (30 µg.^{127}I/100g.), killed 30 sec. - 90 min. after injection of ^{131}I.

Interval after ^{131}I min.	% distribution of ^{131}I *					MIT/DIT	T3/T4
	I	MIT	DIT	T4	T3		
0.5	71.60	9.68	11.71	-	-	0.83	-
1	63.02	13.06	16.79	-	-	0.78	-
2	61.66	15.39	17.48	-	-	0.77	-
5	27.35	28.04	34.57	-	-	0.83	-
10	20.38	30.09	43.10	-	-	0.70	-
20	15.14	34.20	44.9	-	-	0.75	-
40	9.21	32.62	46.49	2.01	0.24	0.72	0.12
60	9.24	32.40	45.21	2.81	0.36	0.72	0.13
90	7.35	31.92	46.00	3.95	0.53	0.69	0.13

* Each value represents the mean of 6 rats.

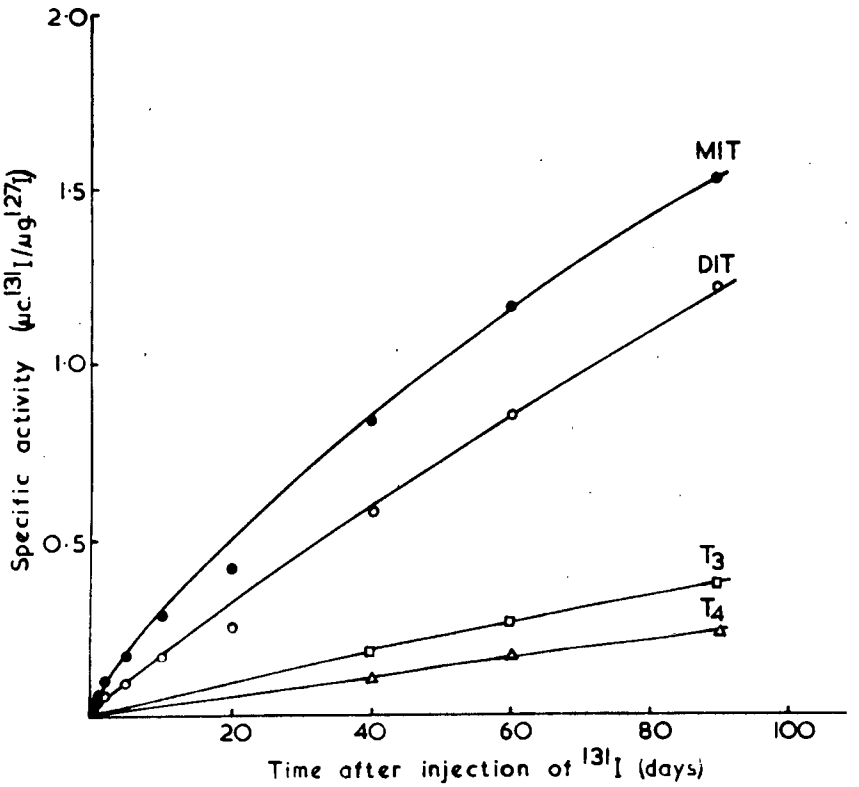

Fig. 19. Variations in absolute specific activity of iodine in the different iodoamino acids of thyroid hydrolysates of rats fed a diet adequate in iodine (30 μg.^{127}I/100g.). Rats killed at intervals 30 sec. - 90 min. after injection of ^{131}I.

TABLE 18.

Absolute specific activity of iodine in the components of thyroid hydrolysates of rats on a normal diet (30 µg.^{127}I/100g.), killed 30 sec. - 90 min. after injection of ^{131}I.

Interval after ^{131}I	Specific activity (µc.^{131}I/µg.^{127}I) *				MIT/DIT	T3/T4
min.	MIT	DIT	T4	T3		
0.5	0.316	0.017	-	-	1.85	-
1	0.551	0.030	-	-	1.83	-
2	0.100	0.054	-	-	1.85	-
5	0.168	0.095	-	-	1.77	-
10	0.291	0.169	-	-	1.72	-
20	0.426	0.256	-	-	1.66	-
40	0.840	0.581	0.101	0.174	1.45	1.73
60	1.172	0.861	0.164	0.276	1.36	1.63
90	1.540	1.229	0.241	0.379	1.24	1.57

* Each value represents the mean of 6 rats.

Similarly, it may be seen from Fig. 19. and TABLE 18. that, from the initial appearance of the iodothyronines, the specific activity of T_3 is always greater than that of T_4.

DISCUSSION

DISTRIBUTION OF INORGANIC IODIDE IN THE THYROID GLAND.

It is well known that the proportion of inorganic ^{131}I increases significantly during enzymic hydrolysis of labelled thyroid homogenates. This increase has been attributed to the deiodination of the labelled iodoamino acids released during the digestion procedure (Tong, Raghupathy and Chaikoff; 1963; Taurog, 1963b; Halmi, 1964). More recently Shimoda (1965) showed that the iodothyronines are deiodinated more readily than the iodotyrosines. Inoue (1966) has suggested than an oxidative step is involved since deiodination is largely inhibited by reducing substances and by anaerobic conditions.

Although the data in TABLE 12 confirm previous reports that enzymic digesion of thyroid homogenates causes extensive deiodination, significant deiodination could be detected during the chromatographic procedures in BAW or BDA solvent systems. It was found that the

% ^{131}I iodide recovered from chromatograms developed in BDA was slightly higher than from chromatograms developed in BAW. These findings could be explicable on either of the following assumptions. (1) that a significant loss of iodide occurred by evaporation from the chromatograms developed in BAW (Schneider and Wolff, 1963) or (2) that deiodination may have occurred to a greater extent in the alkaline solvent than in the acid solvent. If the former possibility were true, then the % ^{131}I iodide recovered from the chromatograms developed in BAW would fluctuate according to the conditions used for drying the chromatograms. However, when the chromatograms which were developed in BAW were dried in a draught the ^{131}I iodide recovery from the chromatograms was not altered.

Wollman and Scow (1955) and Taurog (1963,a) showed that the values for the percentage distribution of thyroidal ^{131}I iodide in unhydrolysed homogenates obtained by paper chromatography are too high due to spontaneous deiodination of thyroglobulin. On the other hand Shimoda (1965) found no significant difference in the values of ^{131}I iodide when determined by electrophoresis and paper chromatography provided that the chromatography paper was not dried for longer than 10 min. The data in TABLE 12. indicate that at the early

intervals after injection similar values for the ^{131}I iodide content of unhydrolysed homogenates were obtained with electrophoresis and paper chromatography in BAW. However, at 48 hr. and 72 hr. significant differences were found, chromatography yielding values 2 - 3 times higher than electrophoresis.

The equilibrium percentages (determined by electrophoresis) reported in the present experiments (0.28% and 0.34%) for the ^{131}I iodide content of unhydrolysed thyroid homogenates, are in good agreement with the values reported by other workers (0.2 - 0.5%) using dialysis (Ingbar and Freinkel, 1958; Halmi and Pitt-Rivers, 1962), precipitation with $Zn(OH)_2$ (Wollman, 1962) or electrophoresis (Rosenberg, Ahn and Chalfen, 1961; Taurog, 1963,b;Simon, 1963).

It is important to realize that, due to the appreciable deiodination occurring during enzymic hydrolysis and paper chromatography, all qualitative and quantitative estimates made of the thyroidal iodoamino acids are incorrect according to the extent of the deiodination occurring during these procedures. In the present experiments relatively high thyroid tissue concentrations (70 - 80 mg./ml.) and high concentrations of thiouracil (0.1M) were used in order to minimize the

deiodination occurring during digestion and chromatography. It was observed by Taurog (1963,a)that the magnitude of the spontaneous deiodination of thyroid extracts varied inversely with the tissue concentration.

It is also possible that the thyroidal iodide values estimated by the various techniques may be on the high side due to contamination with extra iodide derived from the plasma which is present in the gland. However, in one study (Simon, 1963) the sodium space (^{24}Na) of the thyroid was measured and from this it was concluded that the maxium possible contamination by extra-cellular iodide was negligible.

BIOSYNTHESIS OF THE IODOTYROSINES.

It has been widely accepted that the first steps in the biosynthesis of the thyroid hormones is the iodination of the tyrosyl residues in thyroglobulin. This is thought to occur in two steps, first the iodination of tyrosine to form MIT and secondly the iodination of MIT to form DIT. If these steps occur it should be possible to demonstrate a precursor-product relationship between MIT and DIT by isotopic labelling experiments.

Several studies (Michel, 1956; Taurog, Tong and Chaikoff, 1958; Pitt-Rivers and Tata, 1959; Pitt-Rivers,

1962; Ekholm, Zelander and Agrell, 1963) on the distribution of ^{131}I between the iodotyrosines in rat thyroid hydrolysates at different times showed that at early intervals the percentage of the total glandular radioactivity in MIT was greater than that in DIT. Thereafter the ^{131}I content of MIT fell rapidly as the activity in DIT reached a maxium. In these studies the changing $MIT/_{DIT}$ ratios were assumed to reflect changes in the specific activities of the iodotyrosines and consequently were regarded as being consistent with a precursor-product relationship. However, the results of the study on rats fed a diet low in iodine (Chapter 4.) indicate that changes in the percentage distribution of the iodoamino acids may not necessarily reflect changes in absolute specific activity. In rats which had been on an iodine deficient diet for 3 months, the percentage of labelled MIT exceeded that of DIT at early intervals whereas the absolute specific activity of MIT was greater than that of DIT at all times before isotopic equilibrium. It was presumed that the pool sizes of MIT and DIT remained constant during the experiment (at least during the first 6 hr.)

In contrast with the investigations in which changing $MIT/_{DIT}$ ratios were observed, a MIT/DIT ratio

which gradually decreased from 0.83 to 0.50 was found in the present study on rats fed an adequate-iodine diet. An almost identical gradual decrease in the $^{MIT}/_{DIT}$ ratio was reported by Stolc (1962), Plaskett, Barnaby and Lloyd (1963,a) in Sprague-Dawley rats and by Barnaby, Davidson and Plaskett (1965) in rats fed a commercial pellet diet. Several investigators (Bois and Larsson, 1958; De Groot and Davis, 1961,b; Van Zyl and Wilson, 1963; Plaskett, Barnaby and Lloyd, 1963,a; Barnaby, Davidson and Plaskett, 1965) found the $^{MIT}/_{DIT}$ to remain constant in rats. Similarly Kobayashi and Gorbman (1960) reported a constant $^{MIT}/_{DIT}$ ratio in the chick. In addition the <u>in vitro</u> studies of Edelhoch (1962) on the chemical iodination of thyroglobulin are also at variance with the view that a simple precursor-product relationship exists between MIT and DIT.

According to Zilversmit, Entenman and Fishler (1943) for compound A to be the precursor of compound B the following three conditions must hold after a single pulse dose of ^{131}I. (1) The specific activity of A must be greater than B before the latter reaches its maxium specific activity. (2) The specific activity of A must equal that of B when the specific activity of B has reached its maxium specific activity. (3) After the specific activity of B has reached its maxium, the specific

activity of B must be greater than that of A. Although the Zilversmit formulation requires the measurement of the specific activities of the precursor and the product relatively few investigations are available in the literature on the kinetics of labelling of the thyroidal iodoamino acids using absolute specific activity measurements. (Taurog and Chaikoff, 1947; Van Zyl and Wilson, 1963; Rosenberg, Goldman, La Roche and Dimick 1964).

Experimental results conforming with the theoretical requirements of a precursor-product relationship between MIT and DIT, on the basis of relative specific activities, have been reported by Pitt-Rivers (1962). However, Plaskett, Barnaby and Lloyd (1963a) and Barnaby, Davidson and Plaskett (1965) observed that the ratios of the relative specific activities of MIT to DIT remained constant or decreased gradually to unity and were at variance with the anticipated precursor-product relationship between MIT and DIT.

In the present study the ratio of the absolute specific activities of MIT to DIT were found to decrease gradually from 1.85 at early intervals to unity at isotopic equilibrium and therefore were also incompatible with the Zilversmit formulation. In fact the specific activity-time curves indicate rather that the two iodotyrosines were synthesized essentially in parallel. The

ratio of the absolute specific activities of MIT to DIT calculated from the experimental data of Rosenberg et. al. (1964) indicate a similar steady decrease of the ratio from initial values greater than unity to unity at isotopic equilibrium.

Several explanations for the constant ratios of the specific activities of MIT to DIT have been postulated.

(1) De Groot and Davis (1961a, b) suggested that only particular tyrosyl residues in thyroglobulin are mono- and di-iodinated depending upon their position in the thyroglobulin molecule. This specificity of iodination might depend on the steric arrangement in thyroglobulin or on the availability of reactive iodine formed by the peroxidase-iodinase system. In the latter case the MIT/DIT ratio will then be a function of the ratio of reactive iodine to available sites on tyrosine. This hypothesis has been directly tested <u>in vitro</u> by De Groot and Davis (1962) and they found that the MIT/DIT ratio varied inversely with the iodide/tyrosine ratio. The <u>in vitro</u> experiments of Edelhoch (1962) on the iodination of thyroglobulin support the view that the tyrosyl residues are iodinated according to their position in the thyroglobulin molecule.

(2) Plaskett et. al. (1963,a) maintained that thyroidal iodine is heterogenous with respect to MIT and DIT and postulated two iodoprotein pools. One pool was assumed to be small with a very fast turnover and would consist essentially of iodotyrosine-containing thyroglobulin. Coupling of MIT and DIT would lead to the formation of a second iodoprotein pool with a slow turnover and more resistant to proteolysis. A large second iodoprotein pool would result in the gradual accumulation of labelled iodotyrosines and iodothyronines having approximately equal though increasing specific activities. The two-compartment system postulated by Plaskett et. al. (1963,a) is incompatible with the evidence presented by Edelhoch and Lippoldt (1962) that the increase in thyroxyl residues does not render thyroglobulin more resistant to proteolysis.

(3) Rosenberg, Goldman, La Roche and Dimick (1964) suggested that the kinetics of labelling of thyroglobulin are the result of continual intrathyroidal iodination and deiodination reactions which recirculate iodine and lead to randomization of thyroidal iodine. However, the rapid equilibration of the iodotyrosine pool (resulting in synthesis of MIT and DIT of equal specific activity) would necessitate an extremely fast turnover of all the thyroglobulin in the gland. From the absolute rate of

trapping of iodide by the thyroid and the increase in $PB^{131}I$ in rats on adequate diet (Chapter 3, Part I) it is unlikely that thyroglobulin is turned over very rapidly. A rapid turnover of thyroglobulin is also unlikely in view of the large amount present. In addition, Plaskett et. al. (1963,a)have pointed out that rapid equilibration by this mechanism is unlikely because the thyroidal content of labelled iodothyronines progressively increased with time after injection of ^{131}I.

A simpler explanation of the constant or gradually decreasing $^{MIT}/_{DIT}$ ratios can be postulated on the basis of the mechanism of iodination of the tyrosyl and monoiodotyrosyl residues in thyroglobulin. Theoretically the thyroidal iodide, MIT and DIT iodine pools can be considered as individual pools in series. When ^{131}I enters the gland the specific activity of the iodide pool will rise rapidly and then decrease in an exponential manner as the ^{131}I flows from the iodide pool into the MIT iodine pool. The activity in the MIT iodine pool will flow into the DIT iodine pool and from there into the T_4 iodine pool. The activity of the iodine in the MIT and DIT pools will rise and fall in accordance with a precursor-product relationship.

However, in the gland only the thyroidal iodide

pool and the MIT iodine pool can be regarded as being in series. The entrance of additional ^{131}I at the stage of the synthesis of DIT from MIT is likely to obscure any simple precursor-product relationship which exists between these compounds. This is best explained using the reaction mechanism illustrated in Fig. 20 .

For the sake of simplicity the synthesis of MIT and DIT is considered to take place in discrete steps although in reality their synthesis is a continual process.

At the instant ^{131}I enters the gland (Reaction 1) both tyrosyl and pre-existing unlabelled MIT residues in thyroglobulin are labelled at the same time with ^{131}I of the same specific activity x. Consequently both labelled MIT and DIT will be synthesized simultaneously. The newly labelled MIT and DIT is assumed to mix uniformly with the pre-existing MIT and DIT so that the resultant specific activities of the iodine in the MIT and DIT pools are m and d respectively. At some instant later (Reaction 2), more tyrosyl residues and MIT residues of specific activity m are labelled with ^{131}I of the same specific activity x_1. Again the newly labelled MIT and DIT mixes with the pre-existing MIT and DIT so that the resultant specific activities of MIT and DIT pools increase to m_1 and d_1. The specific activity of the MIT and DIT pools will continue to increase until the activity

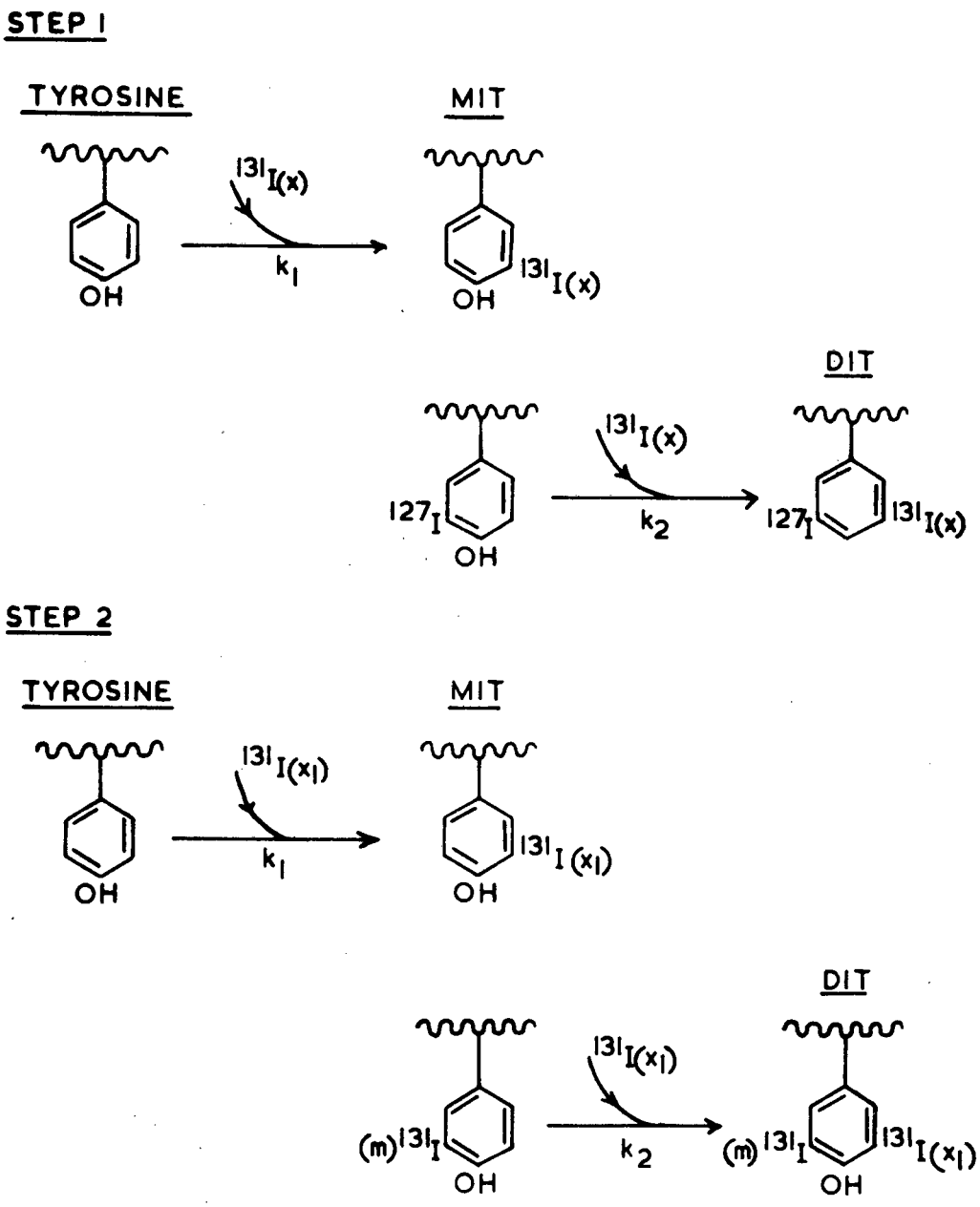

Fig. 20. The proposed scheme of reactions in the iodination of tyrosyl and monoiodotyrosyl residues of thyroglobulin. Letters in parenthese represent specific activities.

leaving the individual pools exceeds that entering. Iodine is continually leaving the MIT and DIT pools by two routes (1) by synthesis of T_3 and T_4 (2) by deiodination of the unbound MIT and DIT.

The proposed scheme of reactions would require the rate constant k_1 and k_2 (Fig. 20.) to be similar. Although in the literature no in vivo measurements of the rate constants k_1 and k_2 have been recorded, it is possible that k_1 and k_2 are similar since the total radioactivities of the MIT and DIT pools vary at a similar rate and since the peak specific activities of the iodine in MIT and DIT occur virtually simultaneously (the DIT peak occurs only slightly later than the MIT peak).

This requirement is in direct contrast with the kinetic studies of Mayberry, Rall and Bertoli (1964) and Mayberry and Rall (1964) in which the rate of iodination of acetyltyrosine (k_1) was found to exceed that of acetylmonoiodotyrosine (k_2) by a factor 30 over the pH range 5.4 to 9.8. However the ratio of the rate constants (k_1/k_2) before correction for the concentration of the ionized forms ranged from 0.91 at pH 5.4 to 9.65 at pH 9.8.

Evidence favouring the possibility of k_1 and k_2 being similar, is the finding of Edelhoch (1965) that

during chemical iodination of native thyroglobulin there was relatively little change in the concentration of MIT but a large increase in the concentration of DIT. The concentration of MIT did not change even after iodination with over 300 moles of iodine. It is apparent from the results of Mayberry et. al. (1964) that the iodine should appear as MIT if the tyrosyl groups in the protein all behaved as in acetyltyrosine. Since Mayberry et. al. (1964) have shown that the ratio k_1/k_2 is independent of pH and ionic strength, environmental factors such as pH or salt concentration could not have accounted for the high DIT/MIT ratio after chemical iodination of thyroglobulin. Consequently it is clear that certain properties of the protein play a role in the distribution of iodine between the tyrosyl and monoiodotyrosyl residues in the thyroglobulin. The reduction in the tyrosyl ultraviolet difference spectrum and the appearance of a diiodotyrosyl difference peak provide evidence that the surface of the protein molecule is involved in the iodination reaction (Van Zyl and Edelhoch, unpublished). In view of the results of the ultraviolet difference spectra, Mayberry (unpublished) has studied the effect of modifying the polarity of the medium on the value of the ratio k_1/k_2. He found a significant fall in this ratio with increasing amounts of methyl alcohol. It is evident therefore that

the ratio k_1/k_2 can be controlled by the local environment of the tyrosyl residues in the thyroglobulin molecule and its value should decrease with increasing shielding of the tyrosyl groups from the surrounding solvent (Van Zyl and Edelhoch, unpublished). When the native organization of thyroglobulin is altered in 8M urea (Edelhoch, 1962) the rates of iodination approach that found by Mayberry et. al. (1964).

Consequently, the structure of the native thyroglobulin appears to be important in controlling the rates of iodination of the tyrosyl and monoiodotyrosyl residues. The relatively high yield of DIT found in native thyroglobulin probably arises from modifications in the rate constants of iodination resulting from tyrosyl interactions with surface groups of the protein which limit the amount of water in the reaction phase (Van Zyl and Edelhoch, unpublished).

On the basis of the above findings it seems reasonable to assume that in the thyroid gland the rate constants k_1 and k_2 are similar. If the rate constants k_1 and k_2 are similar <u>in vivo</u> and if the specific activity of the ^{131}I entering the MIT and DIT pools is also similar, the specific activities of MIT and DIT should vary essentially in parallel as has been shown experimentally in the present study.

It should be possible on the basis of the proposed reaction scheme, to derive an approximate value of the MIT/DIT ratio of the specific activities (S) from the expression,

$$S = \frac{y_1}{y_2} \times \frac{\text{total } {}^{127}\text{I in DIT pool}}{\text{total } {}^{127}\text{I in MIT pool}} \quad \ldots\ldots\ldots \text{(A)}$$

where $\frac{y_1}{y_2}$ = the ratio of the rates of increase in the total absolute radioactivity of the MIT and DIT pools.

The ratio $\frac{y_1}{y_2}$, obtained from the slopes of the semi-logarithmic curves for the initial increase in specific activity of the MIT and DIT iodine pools, was 1.1. Using the value of 1.1 for the ratio $\frac{y_1}{y_2}$, and total ^{127}I values of 2.6 µg. and 5.0 µg. respectively for the MIT and DIT pools, the S ratio was calculated to be 2.12. Hence the S ratio should remain constant at 2.12. However, experimentally it was found to decrease gradually towards unity. Only at very early intervals after injection of ^{131}I was the experimentally determined S ratio (1.85) in agreement with the calculated value.

A plausible explaination for the gradually decreasing S ratio is that glandular deiodination of the free iodotyrosines released after prolealysis of thyroglobulin permits the intrathyroidal reutilization of

iodine. This will lead both to the uniform mixing of the ^{131}I in the MIT and DIT pools and to uniform mixing of the ^{131}I with the ^{127}I so that the S ratio will not remain constant with time but will decrease towards unity. The rate at which the S ratio will decrease towards unity will depend on the turnover times of the MIT and DIT pools and the extent of the intrathyroidal recycling.

Equation (A) above indicates that any changes in the rate of synthesis of MIT or DIT, or in the sizes of MIT and DIT iodine pools will result in alterations in the S ratio. For example, high S ratios will result from a decrease in y_2 or an increase in the size of the MIT iodine pool. A high S ratio may also result if y_2 decreases at a much greater extent than the size of the DIT iodine pool. In fact it was found in rats treated with propylthiouracil that y_2 decreased immediately by an appreciable amount but the size of the DIT iodine pool decreased only gradually (Chapter 5.). On the basis of equation (A) this would result in an increased S ratio which is compatible with the S ratio found experimentally. Similarly, lower S ratios were found to occur in rats fed an iodine-deficient diet (Chapter 4.). In these animals the ratio y_1/y_2 approached unity and the sizes of the MIT and DIT iodine pools became almost equal. Thus it

would be expected from equation (A) that the S ratio would be reduced and would tend towards unity which is again in keeping the experimental S ratio.

In support of this suggestion, both Plaskett, Barnaby and Lloyd (1963a)and Barnaby, Davidson and Plaskett (1965) found S values which remained constant at unity in rats fed a diet low in iodine. Unfortunately they did not report any data concerning the ^{127}I content of the MIT and DIT pools so that the S values could not be calculated from their data.

Assuming the thyroid gland to be in a steady state, it should be possible to determine at any instant the specific activity of MIT and DIT from the following data: (1) The thyroidal trapping rate of iodide. (2) The specific activity of the thyroidal iodide pool. (3) The rates of increase in the total absolute activity of the MIT and DIT pools. (4) The loss of activity from the MIT and DIT pools via hormone synthesis and deiodination.

Preliminary investigations on the calculation of the specific activities of MIT and DIT, using the postulated reaction scheme, indicate that the calculated specific activities of MIT are higher and the calculated specific activities of DIT are lower than those found experimentally. In these calculations the loss of

activity from the iodotyrosine pools was based on the data obtained by Tong, Kerkof and Chaikoff (1962) concerning the deiodination of labelled MIT and DIT by isolated thyroid cells.

The discrepancy between the calculated and the experimentally observed values is probably due to the incorrect assumption of uniform mixing of the newly labelled iodotyrosines with the pre-existing iodotyrosine pools. Recently, much evidence has accumulated which is at variance with the concept of uniform mixing of newly synthesized iodoamino acids with pre-existing pools. Pitt-Rivers and Cavalieri (1963) and Pitt-Rivers (1963) have shown that there are present in thyroglobulin MIT and DIT residues which are different from the remainder. The findings of Pitt-Rivers (1964) have also suggested that the tyrosyl residues iodinated earlier are in a more exposed or accessible location than those which are formed later. Haney and Lissitzky (1963) and Simon, Benabdeljlil and Lissitzky (1964) have shown that labelled thyroglobulin is able to give rise to free labelled MIT and DIT of higher specific activity than that of the average specific activity of the corresponding compounds in peptide linkage. In addition both Schneider (1964) and Rosenberg, La Roche and Ehlert (1966) have shown that thyroidal iodine is heterogenous with respect to turnover.

On the basis of these studies and those of Edelhoch (1962) and Van Zyl and Edelhoch (Unpublished) it seems reasonable to suppose that particular tyrosyl residues in thyroglobulin are preferentially labelled to form MIT of high specific activity. Part of the newly labelled MIT could be selectively labelled, via the scheme of reactions postulated, to form DIT of high specific activity. The consequence would be that the DIT pool would become more highly labelled whereas the MIT pool would be labelled to a lesser extent than expected if the iodine in the iodotyrosine pools was homogeneous with respect to turnover.

It is also possible that the highly labelled MIT and DIT are more easily coupled to form T_3 and T_4 of high specific activity.

Electron microscopy and radioautography have shown that shortly after injection of ^{131}I or ^{125}I ring images appear at the cell-colloid interface. (Leblond and Gross, 1948; Wollman and Wodinsky, 1955; Stein and Gross (1964); Wollman, 1965). This indicates that iodination of thyroglobulin takes place in the lumen of the thyroid follicle, but only at its periphery. Presumably, the activity of the iodinating enzymes is confined to the cell-colloid interface with highest concentration at

the apical membranes of the follicular cells. If the coupling and proteolytic enzymes are within the follicular cell as suggested by the work of Wollman (1965), and because of the relatively slow rate at which the newly iodinated thyroglobulin molecules diffuse from the cell-colloid interface towards the central region of the colloid, these newly labelled thyroglobulin molecules are more apt to undergo coupling and proteolysis. Although these findings are in support of the idea that there is preferential synthesis of highly labelled MIT and DIT which in turn can synthesize T_3 and T_4 of high specific activity, they also indicate that the highly labelled iodoamino acids may not arise only as a consequence of their environment in the thyroglobulin molecule but also as a result of the slow diffusion of thyroglobulin from the site of iodination.

However, it is not certain how the concept of heterogeneity of thyroidal iodine turnover explains the fact that the distribution of the newly acquired ^{131}I in all the iodoamino acids becomes almost identical with that of the pre-existing ^{127}I at such early times after a single injection of ^{131}I. Since the isotopic equilibrium studies of Simon and Morel (1957), Simon (1963) and Wollman (1965) have shown that a period of approximately 50 days is necessary to establish equilibrium between

the specific activity of the diet and that of the thyroidal iodine pools, the injected ^{131}I in the present study could not have become uniformly mixed with the ^{127}I within a few days. Therefore the apparent equilibrium distribution of ^{131}I with that of ^{127}I probably results from a balance struck between the frequency of distribution of large and small follicles, the variation in turnover rate due to follicular size and the distribution of the volume of unlabelled thyroglobulin between the small and large follicles.

BIOSYNTHESIS OF THE IODOTHYRONINES.

a) Thyroxine.

Two theoretically probable routes for the biosynthesis of thyroxine have been proposed, both of which are supported by substantial evidence.

The first hypothesis was suggested by Harington and Barger in 1927. They postulated that T_4 was synthesized in the gland by the coupling of two molecules of DIT with the elimination of an alanine side chain. The second hypothesis proposed by Feuer (1959) regards T_3 as the first iodothyronine to be synthesized and that it gives rise to T_4 by the addition of one atom of iodine.

Several chemical studies have supported the hypothesis of Harington and Barger (1927). Ludwig and von Mutzenbecher (1939) showed that the chemical iodination of proteins, such as casein and serum albumin led to the formation of peptide linked T_4. The incubation of free DIT in a highly alkaline medium was also found to result in the formation of small quantities of T_4 (von Mutzenbecher, 1939; Harington and Pitt-Rivers, 1939). Subsequently, Pitt-Rivers (1948) showed that by protecting both the amino and carboxyl groups of DIT the yield of T_4 could be greatly increased. In addition, the optimal pH for the reaction was in the physiological range (pH 7.5).

Evidence that DIT is the biological precursor of T_4 has been obtained from the <u>in vivo</u> studies of Taurog and Chaikoff (1947), Pitt-Rivers (1962), Pitt-Rivers and Cavalieri (1964) and Rosenberg, Goldman, La Roche and Dimick (1964). The experiments on the rat (Taurog and Chaikoff, 1947) and on the hamster (Pitt-Rivers and Cavalieri, 1964) showed that the specific activity of the iodine in DIT was higher than that of T_4 at early intervals after injection but the specific activity of T_4 subsequently exceeded that of DIT. Thus the specific activity-time curves of DIT and T_4 were in accordance with the established criteria for a precursor-product relation-

ship. However, in the study of Taurog and Chaikoff (1947), the DIT and T_4 fractions were not chromatographically homogeneous, the DIT fraction in fact consisted of MIT and DIT whereas the T_4 fraction was made up of T_3 and T_4. Therefore, these results cannot be regarded as definite evidence in favour of DIT being the precursor of T_4.

The data of Pitt-Rivers (1962) and Rosenberg et. al. (1964) on rats indicated that at early intervals after injection the specific activity of DIT exceeded that of T_4 but with increasing time DIT and T_4 gradually became equal. Such a relationship has been regarded as evidence that DIT is the biological precursor of T_4. However, these observations do not preclude the possibility that T_4 may arise by the iodination of T_3. This possibility was taken into account in the study reported by Pitt-Rivers (1962).

Although Pitt-Rivers (1962) found in rats that the relative specific activity of T_3 was greater than that of T_4 before the latter reached its maxium (as would be predicted by the Zilversmit formulation), it was concluded that T_4 arose by the coupling of DIT. Since at early intervals the relative specific activity of MIT was greater than that of DIT, it was argued that if T_3 arose from the coupling of MIT and DIT it would be expected that the specific activity of T_3 would be greater

than that of T_4 at early intervals. However, the possibility that T_3 is the precursor of T_4 was not precluded. In the more recent study by Rosenberg et. al. (1964), the specific activities of T_3 were reported for only one experiment. However, the specific activities of T_3 fluctuated far too widely to eliminate the possibility of T_4 arising from T_3.

Feuer (1959) investigated the pattern of incorporation of ^{131}I into the thyroid hormones of rat blood and showed that for several hours after injection of ^{131}I, the relative specific activity of T_3 exceeded that of T_4 and then fell as the relative specific activity of T_4 rose. These findings were regarded as indicating that the synthesis of T_3 preceeded that of T_4. A similar conclusion was drawn from previous experiments (Feuer and Vekerdy, 1958) in which the distribution of ^{131}I in thyroidal T_3 and T_4 fractions were measured and found to vary in a similar pattern.

In the present study the absolute specific activity-time curves of DIT and T_4 did not indicate a simple precursor-product relationship between DIT and T_4. The absolute specific activity of DIT exceeded that of T_4 for all times up to approximately 5 days when they became equal. Nevertheless, the results were interpreted as evidence favouring the coupling of two molecules of

DIT to form T_4, since at all intervals before isotopic equilibrium the absolute specific activity of T_3 was greater than that of T_4. If T_4 arose via the iodination of T_3, the specific activity of T_3 would be expected to be greater at early intervals, whereas at later intervals the specific activity of T_4 would exceed that of T_3. Alternatively, it may even be possible that the specific activity of T_4 would exceed that of T_3 at early intervals, if the T_3 is iodinated with ^{131}I of high specific activity similar to that which iodinates tyrosine and MIT. Therefore, on the basis of these theoretical considerations it seems highly improbable that T_4 is synthesized as the result of the iodination of T_3. A similar conclusion was drawn from a previous study by van Zyl and Wilson (1963) in which the absolute specific activities of the thyroidal iodoamino acids were measured.

It should theoretically be possible to determine by which pathway T_4 is synthesized, by calculating the specific activity of T_4 at isotopic equilibrium for each of the pathways proposed from the specific activities of the supposed precursors.

Consider the two possible pathways of T_4 synthesis:

(1) DIT + DIT $\longrightarrow$ T_4

(2) T_3 + I $\longrightarrow$ T_4

If T_4 is synthesized via pathway (1) and the specific activity of the iodine in DIT is d then the specific activity of the iodine in T_4 would also be expected to be d. On the other hand, if T_4 is synthesized by the iodination of T_3 of specific activity x, then the mean specific activity of the iodine in the newly formed T_4 will be $\frac{3x + y}{4}$, where y is the specific activity of the iodine being incorporated.

Unfortunately in the present experiments, it was not possible to compare the calculated specific activities of T_4 for the two pathways with those measured experimentally because at the later intervals the specific activities of the iodine in all the iodoamino acids were similar.

b) Tri-iodothyronine.

So far two possibilities for the biosynthesis of T_3 have been suggested. The first involving enzymic deiodination of T_4, was proposed by Gross and Pitt-Rivers (1953a, b). Roche, Michel, Michel and Lissitzky (1952) being unable to demonstrate deiodination of T_4 by thyroid slices, concluded that T_3 arose by the coupling of one molecule of MIT with one molecule of DIT.

The finding that pig thyroid deiodinase deiodinates T_4 readily, if freed from blood proteins (Tata, 1959) re-opened the possibility that deiodination

of T_4 is a possible pathway of T_3 synthesis. Additional evidence supporting the synthesis of T_3 from T_4 was reported by Plaskett (1961b). Plaskett (1961a, b) investigated the distribution of ^{131}I in the α- and β- rings of T_4 and T_3, and found that both rings in T_3 and T_4 were equally labelled. These findings were considered to be compatible with the view that T_3 was formed from T_4 by deiodination. It would be expected that if T_3 was derived from T_4 , the iodine on both rings of T_3 would have the same specific activity as that of T_4 because the T_4 was originally synthesized by the coupling of two molecules of DIT having the same specific activity.

Pitt-Rivers (1962) found the estimated specific activities of T_3 to be greater than that of T_4 at early intervals. She concluded that T_3 was formed by the coupling of MIT and DIT since the relative specific activity of T_3 exceeded that of T_4 at a time when the relative specific activity of MIT was greater than that of DIT. Plaskett, Barnaby and Lloyd (1963,b) ascribed the apparently high specific activity of T_3 to incomplete chromatographic resolution of T_3 from a highly labelled unidentified substance with a faster turnover. Plaskett et. al. (1963,b) found that when the T_3 and T_4 fractions were resolved by two-dimensional chromatography (butan-1-ol: dioxan : 2N-NH_3 for the first development and

butan-1-ol : 9N-NH_3 for the second development), the ratio of labelled T_3 to labelled T_4 was the same at 15 min. after injection as at 24 hr. Therefore it was concluded that neither T_3 nor T_4 is a major precursor of the other. The findings that the $^{T3}/_{T4}$ ratio remained constant is not entirely unexpected because in this study the relative specific activities of MIT and DIT were reported to be equal throughout the duration of the experiment. However, since both the ratio of $^{T3}/_{T4}$ and $^{MIT}/_{DIT}$ remained constant during the entire period of the experiment, the results were regarded as being compatible with the view that T_3 is synthesized by the coupling of MIT and DIT.

The work of Plaskett (1961b) showed that it was theoretically possible to determine the pathway of synthesis of T_3 by measuring the distribution of the ^{131}I between the two rings in T_3. However, Plaskett was not able to test this possibility since the relative specific activities of MIT and DIT were found to be equal (Plaskett, Barnaby and Lloyd, 1963,b). In more recent studies, Plaskett and Barnaby, (1964), and Barnaby, Davidson and Plaskett (1965) were able to produce a situation in which the relative specific activity of MIT was greater than that of DIT. Using the diazo-coupling technique of Plaskett (1961b), it was possible to show that the iodine on the

β - ring of T_3 was more heavily labelled than the iodine on the α - ring. Since in a previous study (Plaskett, 1961a) it was found that T_4 was equally labelled on the two rings at all intervals after injection, it was concluded the T_3 could not have arisen from T_4 but must have been formed by the coupling of MIT and DIT. It should be pointed out that the evidence for eliminating T_4 as a possible precursor of T_3 was based on separate experiments in which the specific activities of MIT and DIT were actually identical. The distribution of the label in T_4 should also have been determined in the situation where the specific activity of MIT and DIT were different in order to eliminate any possibility of preferential labelling in the β - ring of T_4.

The absolute specific activity data illustrated in Fig. 17. support the hypothesis that T_3 is synthesized by the coupling of MIT and DIT. The absolute specific activity of T_3 was observed to be always greater than that of T_4 until 5 days when specific activities of both became equal. It was therefore considered unlikely that T_3 could have arisen by deiodination of T_4 because under these circumstances the specific activities of T_3 and T_4 would be expected to be equal or that a precursor-product relationship would exist between T_4 and T_3. Additional evidence compatible with the view that T_3 is synthesized

by the coupling of MIT and DIT, is given by the observation that the absolute specific activity of T_3 exceeds that of T_4 at all times when the absolute specific activity of MIT exceeds that of DIT. Also the ratios of the specific activities $^{T3}/_{T4}$ and $^{MIT}/_{DIT}$ were found to decrease at almost the identical rate (TABLE 16).

In the interpretation of the present results the suggestion of Plaskett, Barnaby and Lloyd (1963,b), that the apparently high specific activity of T_3 is ascribed to incomplete chromatographic resolution of T_3 from a highly labelled unidentified substance with a faster turnover, must be considered. From the data of Plaskett et. al. (1963,b) it may be seen that 10 min. after injection the supposed contaminant accounted for approximately 38% of the total radioactivity of the T_3 fraction whereas after 24 hr. it accounted for only 7% of the radioactivity. In addition the data reported by Plaskett et. al. (1963,b) concerning the ratios of $^{T3}/_{T4}$ obtained by one-dimensional chromatographic analysis in BDA, show very little change in the $^{T3}/_{T4}$ ratio from 8 hr. to 24 hr. This indicates that the supposed contaminant is of relatively minor importance after 8 hr. According to these results it seems unlikely that in the present study the supposed contaminant would account entirely for the specific activity of T_3 being greater than that of

T_4 during the interval 24 - 120 hr. In fact the ratio of the specific activity of T_3 to that of T_4 was 1.09 at 24 hr. which is only 25% less than the value of the ratio at 40 min. It is therefore felt that even if the T_3 fraction was contaminated with an unknown compound of fast turnover, the contaminant would play a relatively minor role in causing the specific activity of T_3 to be greater than that of T_4, particularly for periods greater than 8 hr. after injection. Consequently in the present study, since the ratios of the specific activity of T_3 to that of T_4 were greater than unity for all times up to approximately 5 days, it was concluded that T_3 was formed in the thyroid gland by the coupling of MIT and DIT.

Similarly as for T_4, it should be possible theoretically to determine by which pathway T_3 is formed simply by calculating the specific activity of T_3 at isotopic equilibrium for each of the proposed pathways, from the specific activity of the suspected precursors.

Consider the two possible pathways of synthesis of T_3:

(1) $T_4 \longrightarrow T_3 + I$

(2) MIT + DIT $\longrightarrow T_3$

Thus, if T_3 is synthesized by pathway (1) and the specific activity of the iodine in DIT is d, it would be expected that both the specific activity of the

iodine in T_4 and T_3 would be d. If however, T_3 arose via the coupling of MIT and DIT the mean specific of the iodine in T_3 would be expected to be $\frac{2d + m}{3}$ where d and m are the specific activities of DIT and MIT respectively. As in the case of T_4 it was impossible to compare the specific activities of T_3 calculated for the different pathways with those measured experimentally because at the later intervals after injection the specific activity of the iodine in all the iodoamino acids was similar.

CHAPTER 4.

INTRATHYROIDAL IODINE METABOLISM IN RATS FED A DIET LOW IN IODINE.

INTRODUCTION.

Several investigators have reported that after administration of ^{131}I to rats, either the ratio S, of the specific activities of MIT to DIT (Pitt-Rivers, 1962) or the ratio R, of the relative amounts of labelled MIT to DIT (Michel, 1956; Taurog, Tong and Chaikoff, 1958; Pitt-Rivers and Tata, 1959; Pitt-Rivers, 1962) are in accordance with a precursor-product relationship. However, it was found previously (Chapter 3 , Part II) for rats on an adequate iodine diet that both the R and S values decreased gradually with time and were at variance with the Zilversmit formulation. For this reason it was thought possible that varying R or S values in accordance with a precursor-product relationship may be produced by altering the iodine content of the diet, since Leloup and Lachiver (1955), Querido, Schut and Terpstra (1957), and Bois and Larsson(1958) had shown increased R values in rats fed a low iodine diet. Galton and Pitt-Rivers,(1959) have also reported increased R values in rats fed a diet

METHODS

(For detailed descriptions of the procedures refer to Chapter 1 .)

EXPERIMENT 1 : Rats killed at intervals of 3 - 120 hr. after injection of ^{131}I.

Male Sprague-Dawley rats weighing 296 ± 11g. were used. The animals were fed a diet (manufactured by Vereeniging Milling Co.) low in iodine (7.1 ± 0.8 µg./100g.) for 12 weeks and were given demineralized water to drink. The daily intake of iodine was estimated at 1 - 2 µg. per rat.

Approximately 40 µc. carrier-free $Na^{131}I$ was injected intraperitoneally into each rat and thereafter the animals were killed at intervals ranging from 3 - 120 hr. At each interval six rats were killed, their thyroids were removed and counted for uptake of the injected ^{131}I. The thyroid glands were homogenized, hydrolysed with a mixture of trypsin and pancreatin, and the iodoamino acids of the hydrolysates were fractionated by ascending paper chromatography in BDA and BAW. The distribution of ^{131}I and ^{127}I between the various iodinated amino acids was measured. The total thyroidal iodine content was determined by analysis of 25 µl. portions of the thyroid

hydrolysates of rats killed at 72 hr. and 120 hr. The concentrations of PBI and free iodide were measured in the blood of rats killed at 72 hr. and 120 hr.

The distribution of ^{131}I and ^{127}I iodide in the thyroid homogenates of rats killed at 48 hr. and 72 hr. after injection was measured using electrophoresis. The inorganic iodide fractions were extracted with 2.5 ml. KOH (3.57 ml. 4N-KOH made up to 50 ml. with water) and 1 ml. portions of these extracts were analysed for stable iodine.

EXPERIMENT 2 : Rats killed at intervals 30 sec. - 30 min. after injection of ^{131}I.

Male Sprague-Dawley rats weighing 299 $\pm$ 7g. were used. The animals were maintained on the same diet as those used in Experiment 1.

Approximately 170 µc. carrier-free $Na^{131}I$ was injected intravenously into each rat and the rats were killed at intervals 30 sec. - 30 min. after injection. When the interval between injection and autopsy was 5 min. or less, the rats were kept under ether anaesthesia for the entire period with their thyroids partly exposed (see Chapter 3 , Part II, METHODS). At each interval six rats were killed, their thyroids were immediately

removed and plunged into boiling thiouracil, homogenized and boiled for 3 min. The boiled homogenates were hydrolysed enzymically and the distribution of ^{131}I and ^{127}I between the various thyroidal iodoamino acids was measured.

RESULTS.

EXPERIMENT 1 : Rats killed at intervals 3 - 120 hr. after injection of ^{131}I.

Uptake of ^{131}I.

The uptake of ^{131}I by the thyroid gland reached a maximum of 38.8% of the injected dose at 12 hr. and fell to 11.0% after 120 hr. The rate of ^{131}I iodide trapping was determined from the fixation curve (Fig. 21 (b)) and found to be 34.6%/hr. of the injected dose. The release of ^{131}I from the thyroid is shown in Fig. 21 (a). The release curve was found to be biphasic in nature, the biological half-life ($T_{\frac{1}{2}}$) of the thyroidal ^{131}I being 1.7 days for the first exponential and 4.25 days for the second exponential.

Distribution of ^{131}I in the thyroid gland.

a) Unhydrolysed glands.

The distribution of ^{131}I in unhydrolysed thyroid homogenates of rats killed at 72 hr. and 120 hr.

excessive in iodine but the change in the R values was found to be only temporary.

In addition, it was important to produce conditions in which the specific activities of MIT and DIT were very different so that it would be possible to calculate the specific activities of T_4 and T_3 for the various biosynthetic pathways which have been postulated and to compare the calculated values with the values obtained experimentally Chapter 3, Part II, Discussion).

The experiments to be described were undertaken in an attempt to produce a situation where the specific activity of MIT would be greatly different from DIT. Rats were fed a diet low in iodine and the kinetics of labelling of the various thyroidal iodoamino acids were investigated using (1) absolute specific activity measurements and (2) the distribution of ^{131}I between the iodoamino acids. The rats were killed at various intervals after injection, (a) at 3 - 120 hr. and (b) at 30 sec. - 30 min.

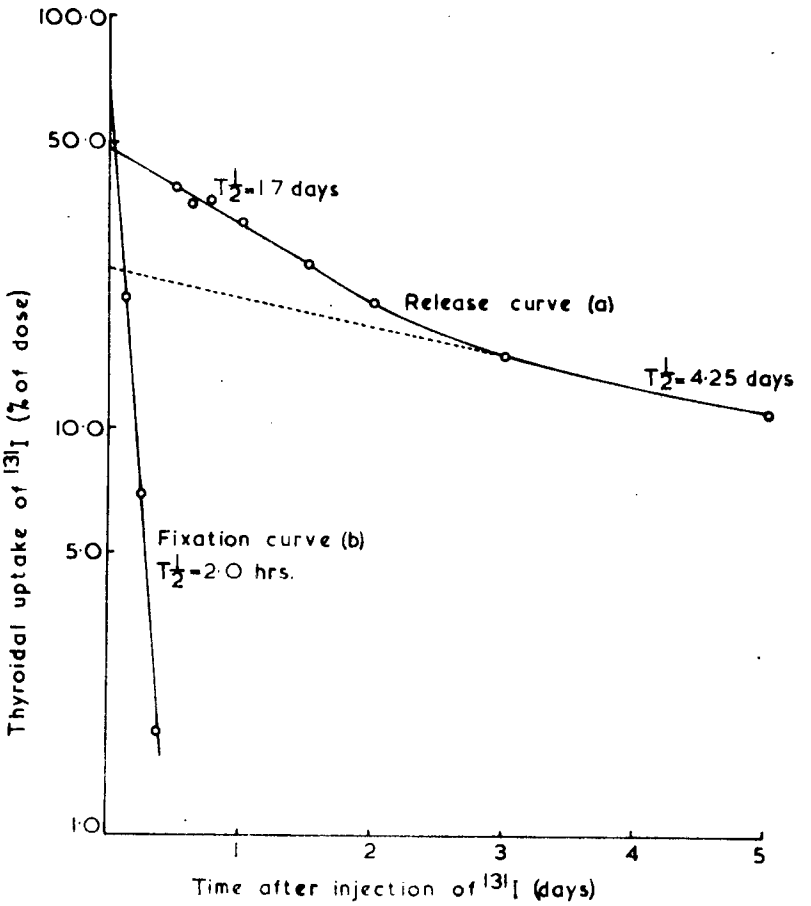

Fig. 21. Curve (a): Release of ^{131}I from the thyroid glands of rats on a low-iodine diet (7 µg.^{127}/100g.).

Curve (b): Rate of fixation of ^{131}I by the thyroid glands of rats fed a diet low in iodine (7 µg.^{127}/100g.).

after injection was determined by electrophoresis. The inorganic ^{131}I iodide accounted for only 0.11% and 0.13% of the total thyroidal ^{131}I at 72 hr. and 120 hr. respectively.

b) Hydrolysed glands.

The percentage of the ^{131}I of the hydrolysate which was present at the origin of the chromatogram increased with time after administration of ^{131}I. At the early intervals it represented 4 - 5% of the total radioactivity on the chromatogram whereas at the later intervals it accounted for approximately 8% of the total activity.

The distribution of ^{131}I between the various iodoamino acids in the thyroids of rats killed 3 - 120 hr. after administration of ^{131}I is shown in Fig.22 . The data used in plotting the curves illustrated in Fig. 22. are presented in TABLE 19. As in the case of rats on a normal iodine diet, the percentage of the total thyroidal ^{131}I present as iodotyrosines always exceeded that of the iodothyronines. MIT and DIT were labelled exceedingly rapidly and had already reached their peak value before the first time interval of the experiment. T_3 and T_4 were labelled more slowly and became maximally labelled only after 24 hr. The equilibrium percentage distribution

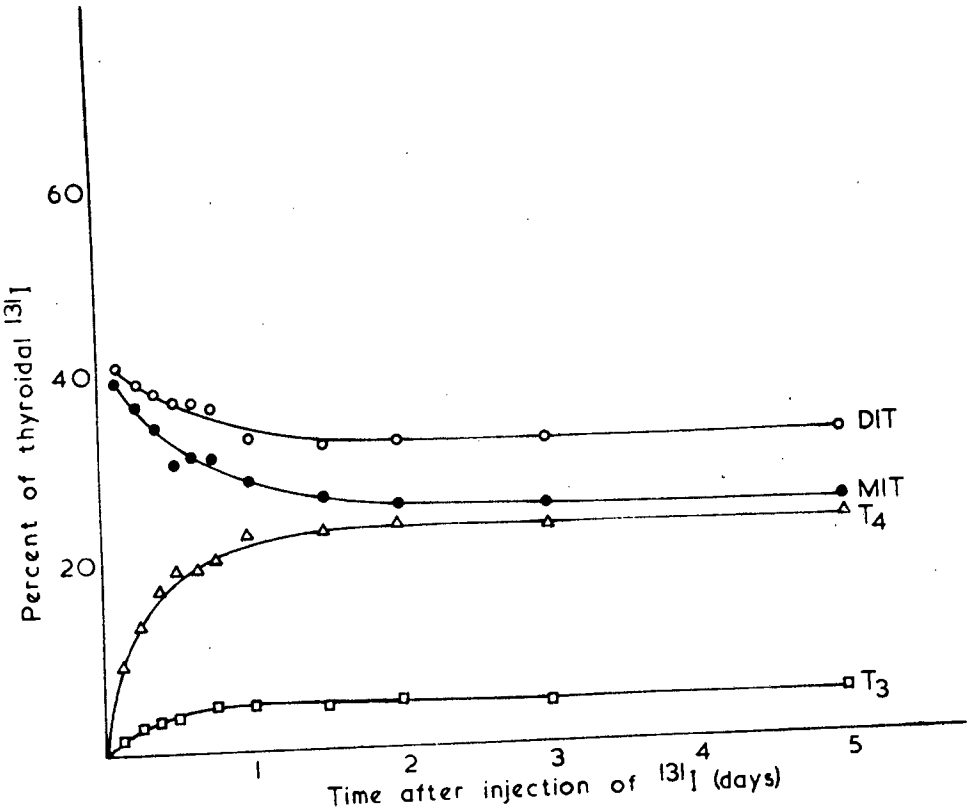

Fig. 22. Distribution of ^{131}I between the various iodoamino acids in thyroid hydrolysates of rats on a low-iodine diet (7 µg.^{127}I/100g.) killed 3 - 120 hr. after injection of ^{131}I.

TABLE 19.

The distribution of ^{131}I between the components of thyroid hydrolysates of rats on a diet low in iodine (7 µg.^{127}I/100g.), killed 3 - 120 hr. after injection of ^{131}I.

Interval after ^{131}I hr.	% distribution of ^{131}I *					MIT/DIT	T3/T4
	I	MIT	DIT	T4	T3		
3	4.21	39.58	41.04	9.27	1.42	0.96	0.15
6	3.92	37.01	39.43	13.70	2.71	0.94	0.20
9	3.50	34.52	38.37	17.19	3.41	0.90	0.20
12	4.62	30.62	37.26	19.50	3.72	0.82	0.20
15	4.82	31.51	37.16	19.68	3.75	0.85	0.19
18	3.80	31.22	36.49	20.03	4.91	0.86	0.25
24	5.11	28.34	33.65	22.99	4.71	0.84	0.20
36	5.52	26.86	32.34	23.01	4.37	0.83	0.19
48	5.80	25.83	32.50	23.92	4.81	0.80	0.20
72	6.17	25.35	32.31	23.06	4.20	0.78	0.18
120	6.75	24.97	32.40	23.11	4.52	0.77	0.20

* Each value represents the mean of 6 rats.

of ^{131}I in MIT, DIT, T_4 and T_3 was 25.1, 32.4, 23.1 and 4.5 respectively.

The MIT and DIT both diminished significantly during the period 3 - 24 hr. and thereafter approached the apparent equilibrium values. The reduction in labelled MIT and DIT during the first 24 hr. corresponded with the relative increase in labelled T_3 and T_4. The decrease in percentage label during the period was much greater for MIT than DIT. Although for all the time intervals investigated the percentage of ^{131}I in DIT exceeded that in MIT, it can be seen from the shape of MIT and DIT curves in Fig. 22. that it is extremely likely that the radioactivity in MIT will exceed that in DIT at intervals earlier than 3 hr.

The ratio R, of labelled MIT to labelled DIT decreased from approximately unity at 3 hr. to 0.77 at 120 hr. whereas the ratios of labelled T_3 to T_4 remained constant at approximately 0.2 for the entire duration of the experiment (TABLE 19).

Distribution of ^{127}I in the thyroid glands.

a) Unhydrolysed glands.

The amount of stable iodine was determined in the inorganic and organic iodine fractions separated by electrophoresis of unhydrolysed thyroid homogenates of

rats killed at 36 hr. and 48 hr. Analyses of 12 glands gave a value of 0.0052 ± 0.0018 µg. iodide which is equivalent to 0.13% of the mean total iodine of the gland (4.043 µg.)

b) Hydrolysed glands.

The quantitative and relative distributions of the thyroidal iodine and the thyroidal iodoamino acids measured in the thyroid hydrolysates of rats killed at 48 and 72 hr., after injection, are shown in TABLE 20 . The mean recovery of ^{127}I after chromatography, based on the total iodine content of portions of unchromatographed hydrolysates was 91%.

The origin material accounted for approximately 8% of the total thyroidal ^{127}I present in the hydrolysates of rats killed at 48 hr. and 72 hr. Since the origin material accounted for such a large fraction of the total thyroidal iodine, the molar ratios of the iodoamino acids were calculated on the assumption that the origin material (presumably unhydrolysed thyrogolubin) had the same composition as the hydrolysed thyroglobulin. This assumption may not be strictly correct as Barnaby, Davidson and Plaskett (1965) found that the radioactivity of the origin could be related to the content of labelled iodothyronines in the gland. However, the molar ratios of MIT : DIT : T_4 : T_3 when based on the above assumption were 20 : 14 :

TABLE 20.

The distribution of ^{127}I and of iodine containing compounds in the thyroid glands of rats on a diet low in iodine (7 µg. ^{127}I/100g.)

	Stable iodine content/gland *		Iodoamino acid content/gland *	
	ug.	%	ug.	%
Total I	4.043	100.00	-	-
MIT	1.057	26.17	2.563	37.8
DIT	1.322	32.75	2.259	33.3
T4	0.985	24.40	1.511	22.3
T3	0.145	3.59	0.250	3.7
Iodide (electrophoresis)	0.005 **	0.13**	0.005	0.1
Iodide (chromatography)	0.191	4.73	0.191	2.8
Origin	0.314	7.78	-	-
Other	0.278	0.69	-	-
MIT/DIT	0.80		1.13	
T3/T4	0.15		0.17	

* Each value represents the mean of 12 rats.

** Determination of the thyroidal iodide content using electrophoresis was performed on 12 rats.

5 : 1.

From TABLES 19 and 20. it may be seen that the relative distributions of the ^{127}I and ^{131}I became almost identical within 24 - 36 hr. after injection. Hence within a very short time the newly acquired ^{131}I had an equilibrium distribution almost identical with that of the pre-existing ^{127}I.

Absolute specific activity of the iodoamino acids.

The changes in the absolute specific activities ($\mu c.^{131}I/\mu g.^{127}I$) of the various thyroidal iodoamino acids were measured as a function of time (Fig. 23 and 24.) The data presented in TABLE 21. were used to construct the curves illustrated in Fig. 23 and 24.

The specific activity-time curves (Fig. 23 and 24.) did not show any simple precursor-product relationship between the iodotyrosines or the iodothyronines. As in the rats on a normal iodine diet the specific activity of MIT exceeded that of DIT, but for a much shorter time.

The specific activities of MIT and DIT became equal at approximately 2 days and remained equal for the duration of the experiment. The peak specific activity of MIT occurred between 9 - 12 hr. whereas the peak specific activity of DIT was slightly later occurring between 12 - 15 hr. The specific activities of both MIT and DIT exceeded that of T_4 except after 2 days when the specific activities of all three iodoamino acids became equal and remained equal.

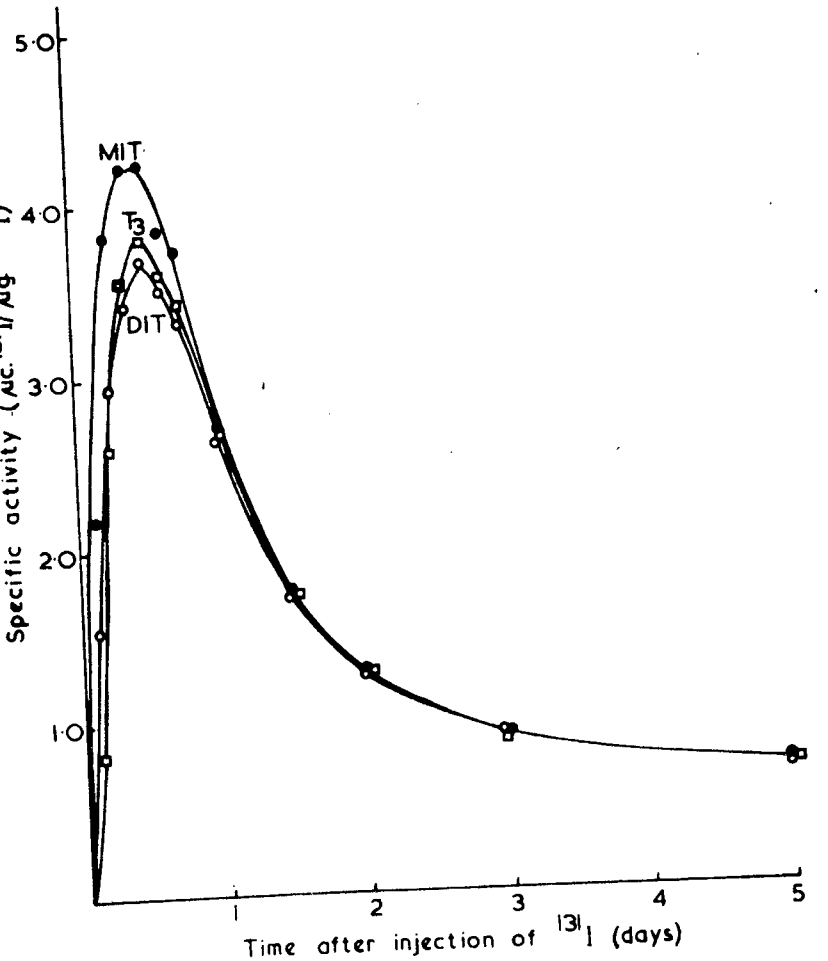

Fig. 23. Absolute specific activities of the iodine in MIT, DIT and T_3 in thyroid hydrolysates of rats fed a low-iodine diet (7 μg. ^{127}I/100g. Rats killed at intervals 3 - 120 hr. after injection of ^{131}I.

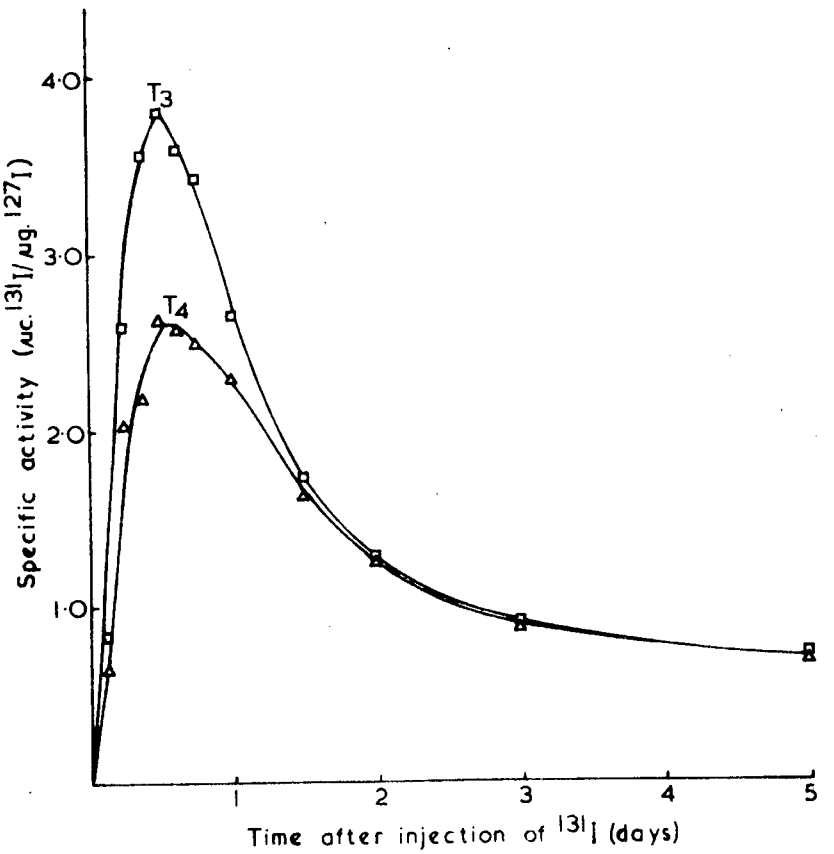

Fig. 24. Absolute specific activities of the iodine in T_3 and T_4 in thyroid hydrolysates of rats fed a low-iodine diet (7 μg.^{127}I/100g.). Rats killed at intervals 3 - 120 hr. after injection of ^{131}I.

TABLE 21.

Absolute specific activity of iodine in the components of thyroid hydrolysates of rats on a diet low in iodine (7 μg.^{127}I/100g.), killed 3 - 120 hr. after the injection of ^{131}I.

Interval after ^{131}I hr.	Specific activity (μc.^{131}I/μg.^{127}I)*				MIT/DIT	T3/T4
	MIT	DIT	T4	T3		
3	2.18	1.57	0.63	0.82	1.39	1.30
6	3.82	2.93	2.01	2.58	1.30	1.28
9	4.22	3.41	2.17	3.55	1.24	1.33
12	4.24	3.68	2.62	3.80	1.15	1.20
15	3.85	3.49	2.57	3.59	1.10	1.13
18	3.73	3.31	2.49	3.42	1.13	1.15
24	2.70	2.63	2.28	2.65	1.03	1.02
36	1.75	1.73	1.61	1.73	1.03	1.07
48	1.29	1.26	1.26	1.27	1.02	1.01
72	0.90	0.90	0.89	0.90	1.00	1.01
120	0.70	0.69	0.70	0.71	1.01	1.01

* Each value represents the mean 6 rats.

The specific activity of T_3 was found to exceed that of DIT at 6 hr. and remained greater than DIT until isotopic equilibrium was reached. At the early intervals the specific activity of T_3 increased more rapidly than that of T_4 with the consequence that the specific activity of T_3 was at all times up to 2 days greater than that of T_4.

Both the S ratio and the ratio of the specific activities of T_3 to T_4 decreased gradually to unity at approximately the same rate. At 3 hr. the S and T_3/T_4 ratios were 1.39 and 1.30 respectively.

EXPERIMENT 2 : Rats killed at intervals 30 sec. - 30 min. after injection of ^{131}I.

Because in the above study (3 hr. - 120 hr.) it seemed likely from the relative distributions of MIT and DIT that at the early intervals the amount of labelled MIT may exceed that of DIT, it was of interest to study in detail the kinetics of labelling at the early intervals.

Distribution of ^{131}I in hydrolysed glands.

The pattern of labelling of MIT and DIT for the interval 30 sec. - 30 min. is shown in Fig. 25 . The data used to construct these curves are presented in TABLE 22.

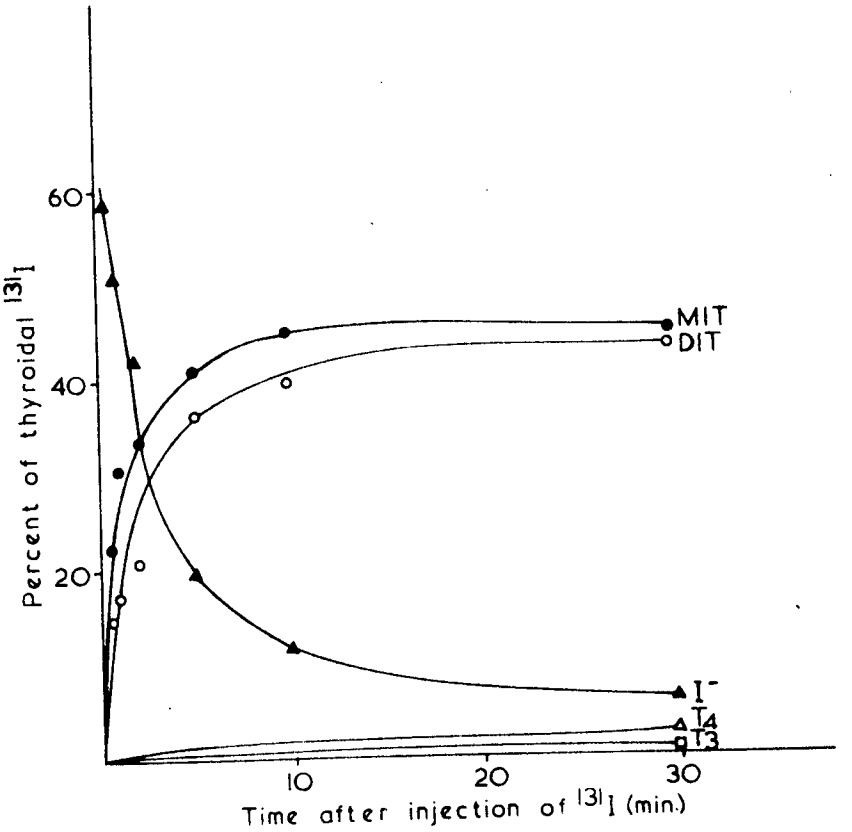

Fig. 25. Distribution of ^{131}I between the various iodoamino acids in thyroid hydrolysates of rats on a low-iodine diet (7 µg.^{127}I/100g.) killed 30 sec. - 30 min. after injection of ^{131}I.

TABLE 22.

The distribution of ^{131}I between the components of thyroid hydrolysates of rats on a diet low in iodine (7 µg.^{127}I/100g.), killed 30 sec. - 30 min. after injection of ^{131}I.

Interval after ^{131}I min.	% distribution of ^{131}I *					$MIT/_{DIT}$	$T3/_{T4}$
	I	MIT	DIT	T4	T3		
0.5	58.50	22.22	14.51	-	-	1.53	-
1	51.89	25.23	17.01	-	-	1.48	-
2	42.00	33.75	20.62	-	-	1.15	-
5	19.48	41.02	36.03	-	-	1.14	-
10	11.45	44.90	39.58	-	-	1.13	-
30	5.90	44.37	43.10	2.49	0.51	1.03	.20

* Each value represents the mean of 6 rats.

The ^{131}I was incorporated into organic linkage very rapidly and after 30 sec. 37% of the total thyroidal ^{131}I was already protein-bound in the form of MIT and DIT. The relative abundances of labelled MIT and DIT became maximal at 10 min. after injection.

During the entire period of the study the amount of labelled MIT exceeded that of labelled DIT. The R ratio was 1.53 at 30 sec. and gradually decreased to 1.03 at 30 min. Labelled T_4 was first detected at 10 min. after injection but was not analysed. After 30 min. both T_3 and T_4 were present and at this interval the ratio of labelled T_3 to labelled T_4 was 0.20.

Absolute specific activity of the iodoamino acids.

At all times the absolute specific activity of the iodine in MIT exceeded that in DIT (Fig. 26). The S ratio was 1.46 at 30 sec. and decreased to only 1.39 at 30 min. (TABLE 23). Similarly it may be seen from TABLE 23 that from the initial appearance of the iodothyronines the specific activity of T_3 was greater than that of T_4, the ratio of the specific activities of T_3 to T_4 at 30 min. was 1.44.

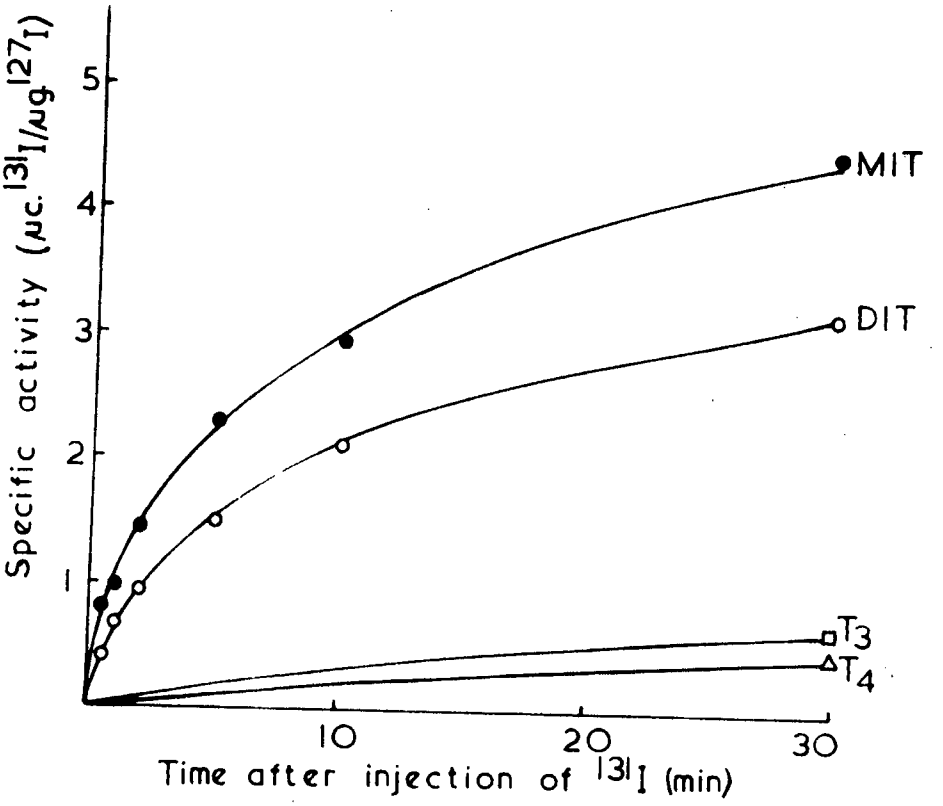

Fig. 26. Variations in absolute specific activity of iodine in the different iodoamino acids of thyroid hydrolysates of rats fed a low-iodine diet (7 μg. ^{127}I/100g). Rats killed at intervals 30 sec. - 30 min. after injection of ^{131}I.

TABLE 23.

Absolute specific activity of iodine in the components of thyroid hydrolysates of rats on a diet low in iodine (7 µg./100g.), killed 30 sec. - 30 min. after injection of ^{131}I.

Interval after ^{131}I min.	Specific activity (µc.^{131}I/µg.^{127}I)*				MIT/DIT	T3/T4
	MIT	DIT	T4	T3		
0.5	0.60	0.41	-	-	1.46	-
1	0.98	0.67	-	-	1.46	-
2	1.42	0.95	-	-	1.49	-
5	2.19	1.51	-	-	1.46	-
10	2.96	2.10	-	-	1.41	-
30	4.41	3.18	0.43	0.62	1.39	1.44

* Each value represents the mean of 6 rats.

DISCUSSION.

Trapping and release of iodine by the gland.

Judging from the rate of iodine trapping and release from the glands of rats fed a low iodine diet, the turnover rate is greatly increased. The thyroidal ^{131}I was found to accumulate at a rate of 34.6%/hr. of the injected dose whereas in rats on an adequate iodine diet, ^{131}I accumulated at a rate of only 14.1%/hr. of the injected dose. The rate constant k_T was calculated from the maxium thyroidal uptake of ^{131}I (Umax) and was found to be 0.134/hr. Using the value of k_T, knowing the concentration of serum ^{127}I (0.10 µg./100 ml.) and assuming the iodide space to be approximately the same as in rats on an adequate iodine diet, the absolute rate of iodine trapping was calculated to be 0.79 µg./day in rats on a low iodine diet. Consequently the daily absolute trapping rate was only 45% of that of animals on an adequate iodine diet. Hence the thyroid gland did not compensate for the low intake of dietary iodine by increasing the uptake of iodine. On the contrary, Rosenberg et. al. (1964) found the absolute trapping rates of rats fed normal and low iodine diets to be the same.

The ^{131}I release curve was found to be biphasic in nature with an initial rapid phase of disappearance of ^{131}I which then stabilized at a slower rate. As already pointed out (Chapter 3 , Part I, Discussion) this may well be expected in thyroid glands with low iodine stores and having a faster turnover. It is possible that an increase in iodine turnover coupled with an increase in reutilization of iodine derived from peripheral degradation of secreted hormone could cause a noticable stabilization of the release curve at a slower rate. Another possibility which could give rise to a biphasic release curve is that the ratio of the smaller follicles to the larger follicles may increase in iodine deficient glands. This could cause a faster uptake and release of ^{131}I at the earlier intervals than is found in normal glands. Low iodine stores would in fact enhance the effect of the faster turnover of the small follicles. Unfortunately the fraction of ^{131}I secreted as hormone which is reutilized was not measured in the iodine-deficient rats and therefore it was not possible to distinguish between the above suggestions.

Distribution of thyroidal ^{127}I.

Very little data is available in the literature concerning the distribution of ^{127}I in the thyroid gland of

rats fed a low iodine diet. At the commencement of this study the only attempt at measuring the distribution of ^{127}I in iodine deficient glands was that made by Querido, Schut and Terpstra (1957). However, these workers only measured the distribution of ^{127}I in MIT and DIT fractions. Recently Heninger and Albright (1966) measured the distribution of ^{131}I and ^{127}I between the various iodine-containing compounds in the rat thyroid under steady state conditions.

In the present experiments, there was a significant reduction in the concentrations of ^{127}I of all iodoamino acid fractions in the iodine-deficient thyroid. In addition, the distribution of the ^{127}I between the various iodoamino acids was entirely different to that found in rats on an adequate-iodine diet. With iodine deficiency there was an increase in the ratio of MIT to DIT (0.80) and in the ratio of labelled T_3 to T_4 (0.15). The increase in the $^{MIT}/_{DIT}$ ratio was due to a much greater reduction in ^{127}I content of DIT relative to MIT. Similarly, the increase in $^{T3}/_{T4}$ ratio was due to a greater reduction in the relative amount of ^{127}I in T_4. The value of the $^{MIT}/_{DIT}$ ratio obtained by Heninger and Albright (1966) was almost identical to that found in the present study but their ratio of T_3 to T_4 was much higher.

Incorporation of ^{131}I into the iodoamino acids.

The pattern of labelling of the iodotyrosines in iodine deficient animals was different from that of animals on an adequate diet, particularly at early intervals after injection of ^{131}I. In the iodine deficient glands labelled MIT exceeded that of DIT at early intervals but at later intervals labelled DIT exceeded that of MIT. Several investigators (Michel, 1956; Taurog and Chaikoff, 1958; Pitt-Rivers and Tata, 1959; Pitt-Rivers, 1962) have presumed that similar patterns of labelling of MIT and DIT reflect changes in the specific activity of the two pools which are consistent with a simple precursor-product relationship. However, the specific-time curves of MIT and DIT (Fig. 23) do not indicate that the kinetics of labelling of the two iodotyrosines is in accordance with a simple precursor-product relationship. The specific activity of MIT was at all times greater than that of DIT until isotopic equilibrium. Consequently it seems as though changes in the relative distributions of the ^{131}I between the iodoamino acids cannot be assumed to reflect changes in specific activity.

The pattern of labelling found in the present study is at variance with that observed by Leloup and Lachiver (1955) and Querido et. al. (1957). In the former study the labelled MIT exceeded labelled DIT for all inter-

vals from 2 - 72 hr. whereas in the latter study the labelled DIT exceeded that of labelled MIT, but at later intervals the reverse was true. In both the above mentioned studies, butanol extracts of the thyroid hydrolysates were analysed whereas in the present study whole hydrolysates were analysed. It may be possible that butanol extraction of hydrolysates gives rise to an entirely different distribution of ^{131}I from that actually present in the original hydrolysates. Such a possibility could arise due to the difference in solubilities of the iodotyrosines and iodothyronines in butanol with variations in pH (Blau, 1933). In addition, the extraction and drying procedures could cause deiodination of the iodoamino acids. By applying whole thyroid hydrolysates to the chromatographic papers, it was possible to eliminate any possibilities which could cause a variation in the distribution of the iodoamino acids.

The dietary intake of iodine is certainly an important factor in controlling the pattern of labelling of the iodoamino acids. In two other studies in which the dietary iodine content was low the fraction of labelled MIT was greater than that of DIT at early intervals after injection of ^{131}I (Taurog and Chaikoff, 1958; Rosenberg, Goldman, La Roche and Dimick, 1964). The level of dietary iodine which will cause labelled MIT to exceed

labelled DIT seems to be between 14 - 30 µg./100g. In the study of Rosenberg et. al. (1964), in which the dietary iodine content was estimated to be 14 µg./100g., MIT was reported to be greater than DIT whereas in the previous experiments (Chapter 3 , Part II) where the dietary iodine content was 30 µg./100g. DIT was found to be higher.

The equilibrium distribution of the ^{131}I between the different iodoamino acids was markedly different from that observed in rats on an adequate-iodine diet. Both the equilibrium $^{MIT}/_{DIT}$ and $^{T3}/_{T4}$ ratios were increased and therefore confirm the reports of Leloup and Lachiver (1955), Querido, Schut and Terpstra (1957) and Bois and Larsson (1958). In addition, the pattern of labelling is compatible with the view that the T_3 content of the gland depends essentially on the ratio of MIT to DIT (Lachiver and Leloup, 1955).

The kinetics of labelling of the iodoamino acids were not very different from that found in rats on an adequate-iodine diet. However, it was observed that the peak specific activities of all the iodoamino acids and the attainment of isotopic equilibrium occurred much earlier in iodine deficient rats. The initial ratios of the specific activities of MIT to DIT and T_3 to T_4 were also lower. It is possible to account for

the decrease in the S ratio on the basis of the mechanism of labelling of the iodotyrosines suggested previously in Chapter 3, Part II. Judging from the rate of change in the specific activities of MIT and DIT, almost as much activity enters the MIT pool as the DIT pool. Assuming that the amounts of ^{131}I entering the MIT and DIT pools are proportional to amounts of ^{127}I entering these pools and substituting the ^{127}I contents of the MIT and DIT pools in equation A (Chapter 3, Part II, Discussion), the S ratio was calculated to be 1.25. This calculated S ratio is in reasonable agreement with the initial experimental value of 1.46.

It is surprising to find from the specific activity data of the present study and of the studies of Rosenberg et. al. (1964) and Barnaby, Davidson and Plaskett(1965), that almost as much activity enters the MIT pool as the DIT pool. This is contrary to what would be expected since the ^{127}I in the DIT pool was found to decrease to a greater extent than the ^{127}I in the MIT pool.

The pattern of the specific activity-time curves of the thyroidal iodoamino acids of iodine-deficient glands is similar to that of normal glands and therefore supports the previous conclusions that T_4 is synthesized by the coupling of two molecules of DIT and T_3 is formed by

the coupling of MIT and DIT (Chapter 3, Part II, Discussion). Additional support for the synthesis of T_3 via the coupling of MIT and DIT is the fact that the specific activity of T_3 in the iodine-deficient rats was found to be greater than both DIT and T_4 but less than that of MIT. The specific activity of T_3 would be expected to be greater than that of DIT and less than MIT if the T_3 is synthesized from MIT and DIT, where the specific activity of MIT exceeds that of DIT.

Similarly as in the case of rats on an adequate iodine diet, it was not possible to compare either the calculated specific activities of T_3 or T_4 for the pathways postulated with those measured experimentally, because at the later intervals the specific activities of all the iodoamino acids again became equal.

CHAPTER 5.

INTRATHYROIDAL IODINE METABOLISM IN RATS TREATED WITH PROPYLTHIOURACIL.

INTRODUCTION.

It has long been known that treatment with propylthiouracil (PTU) and other antithyroid drugs inhibits organic binding of iodine and leads to a reduction in the total iodine in the thyroid gland (Astwood and Bissell, 1944). Recently it has been established that PTU not only inhibits the iodination of tyrosyl and monoiodotyrosyl residues in thyroglobulin but also their coupling to form T_3 and T_4 (Richards and Ingbar, 1959; Mayberry and Astwood, 1960; Iino, Yamada and Greer, 1961). Slingerland, Graham, Josephs, Mulvey, Trakas and Yamazaki (1959) found that the iodination of monoiodotyrosyl residues is more sensitive to PTU than the iodination of tyrosyl residues. On the other hand Iino et. al. (1961) and Schimoda (1964) demonstrated that coupling to form iodothyronines is depressed by smaller amounts of PTU than the other biosynthetic steps.

Although many studies have been reported in the literature on the effects of graded doses of PTU on the intrathyroidal iodine metabolism, the kinetics of

labelling of the thyroidal iodoamino acid pools do not appear to have been studied in rats treated with PTU. Therefore it was considered to be of interest to investigate the kinetics of labelling in PTU-treated rats. In addition, it was hoped to produce a permanent situation in which the specific activity of MIT would be greatly different from DIT so that it would be possible to calculate the specific activities of T_4 and T_3 for the various biosynthetic pathways which have been postulated and to compare the calculated values with the values obtained experimentally (Chapter 3 , Part II, Discussion).

Therefore in the studies to be described the PTU was administered (a) as a single dose 5 min. after injection of ^{131}I and (b) at 48 hr. and 24 hr. before injection of ^{131}I and subsequently every 24 hr. until death. The latter study was undertaken in order to maintain the greatly increased R and S ratios found only in the early time intervals of the former study.

METHODS

(For detailed descriptions of the procedures used, see Chapter 1).

EXPERIMENT 1 : PTU administered 5 min. after injection of ^{131}I.

Male Wistar rats weighing 283 ± 15g. were used. The animals were fed a diet (manufactured by Vereeniging Milling Co.) low in iodine (6.7 ± 0.3 µg./100g.) for 4 weeks and were given demineralized water to drink.

Approximately 300 µc. carrier-free $Na^{131}I$ was injected intraperitoneally into each rat and an uptake of ^{131}I of 5 min. was allowed before injection of 0.5 mg. PTU. The PTU was injected subcutaneously as a fine suspension in 0.5 ml. saline. Groups of six animals were killed at intervals ranging from 2 - 168 hr. after injection of ^{131}I. The thyroid glands were removed, counted for uptake of ^{131}I, and then hydrolysed enzymically. The specific activities of the iodine in the thyroidal iodoamino acids, fractionated from the hydrolysates by paper chromatography in BAW and BDA, were determined. The distribution of ^{131}I between the various iodoamino acids was also measured.

The specific activity of the inorganic iodide in the thyroid homogenates of rats killed 48 - 168 hr. after injection was measured using electrophoresis.

EXPERIMENT 2 : PTU administered before and after injection of ^{131}I.

Male Sprague-Dawley rats weighing 291 $\pm$ 13g. were used. The animals were maintained on a diet adequate in iodine (30 µg./100g.) and had free access to tap water.

Each rat received PTU and ^{131}I according to the following dosage schedule:

0 hr.	:	0.2 mg. PTU
24 hr.	:	0.2 mg. PTU
48 hr.	:	approximately 550 µc. ^{131}I
48 hr. 5 min.	:	0.5 mg. PTU
subsequently every 24 hr. until death	:	0.2 mg. PTU

The ^{131}I was injected intraperitoneally whereas the PTU was injected subcutaneously as a fine suspension in 0.5 ml. saline. Groups of six rats were killed at intervals ranging from 3 - 168 hr. after injection of ^{131}I. The thyroid glands were removed, counted for uptake of the injected ^{131}I, and homogenized in thiouracil in ice. Portions of the homogenate (25 µl. were taken for electrophoresis in order to determine the

specific activity of the thyroidal inorganic iodide. The rest of the homogenate was hydrolysed enzymically. The iodoamino acids present in the hydrolysates were fractionated by ascending paper chromatography in BDA and BAW. The distribution of ^{131}I and ^{127}I between the various iodoamino acids was measured.

RESULTS.

EXPERIMENT 1 : PTU administered 5 min. after injection of ^{131}I.

Uptake of ^{131}I.

The uptake of ^{131}I by the thyroid glands was found to reach a maxium of 3.6% at 2 hr. after injection of ^{131}I and remained approximately at this level for 48 hr. Thereafter it decreased gradually to 1.7% at 168 hr.

Distribution of ^{131}I in the thyroid gland.

The distribution of ^{131}I between the different iodoamino acids in the thyroid hydrolysates of the rats killed 2 - 168 hr. after injection is shown in Fig. 27 . The data for plotting the curves are presented in TABLE 24. At all times the fraction of the total thyroidal ^{131}I present in MIT and DIT exceeded that of T_3 and T_4.

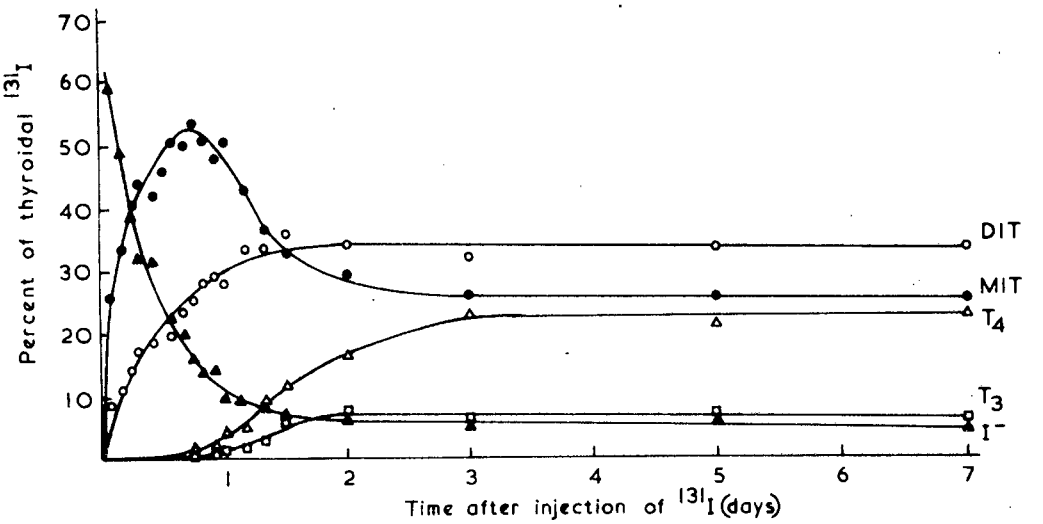

Fig. 27. Distribution of 131I between the various iodoamino acids in thyroid hydrolysates of rats injected with 0.5 mg. PTU 5 min. after administration of ^{131}I. Rats killed at intervals 2 - 168 hr. after injection of ^{131}I.

TABLE 24.

The distribution of ^{131}I between the iodoamino acids of thyroidal hydrolysates of rats injected with 0.5 mg. PTU 5 min. after administration of ^{131}I. The rats were killed at intervals 2 - 168 hr. after the injection of ^{131}I.

Interval after ^{131}I hr.	% distribution of ^{131}I*					MIT/DIT	T_3/T_4
	I	MIT	DIT	T4	T3		
2	58.9	25.6	8.3	-	-	3.08	-
4	39.2	33.4	10.8	-	-	3.09	-
6	38.2	40.5	14.3	-	-	-	-
8	32.4	44.0	17.4	-	-	2.53	-
10	31.6	42.2	18.6	-	-	-	-
14	22.4	50.6	19.6	-	-	2.58	-
16	19.9	50.0	23.4	-	-	2.14	-
18	16.2	53.6	25.3	-	-	2.12	-
20	12.6	51.0	28.3	1.0	0.4	1.80	0.40
22	14.3	48.1	29.2	1.4	0.6	1.65	0.43
24	9.6	50.6	28.0	3.6	1.2	1.81	0.33
28	9.3	43.3	33.5	4.9	1.5	1.29	0.36
32	8.7	36.6	33.5	9.0	2.7	1.09	0.30
36	7.5	32.9	36.0	11.9	5.6	0.91	0.47
48	6.0	29.4	34.1	16.6	7.5	0.86	0.45
72	4.7	26.1	32.2	23.0	6.2	0.81	0.27
120	5.5	25.5	33.9	21.3	6.8	0.75	0.32
168	4.4	25.0	33.1	23.6	5.9	0.76	0.25

* Each value represents the mean of 6 rats.

During the interval 2 - 32 hr., the ^{131}I of MIT greatly exceeded that of DIT and reached maxium labelling at 18 hr. Thereafter the radioactivity in MIT decreased rapidly and dropped below that of DIT at 36 hr. and then approached an apparent equilibrium value of 25% at 72 hr. The labelled DIT increased steadily but much less rapidly than MIT and reached an equilibrium value of 33% at 2 days.

The percentage of radioactivity in T_4 exceeded that of T_3 for the entire duration of the experiment. Both labelled T_3 and T_4 increased extremely slowly during the period 2 - 21 hr. but thereafter the rate of synthesis of labelled T_3 and T_4 increased significantly, probably as the effect of the PTU wore off, and reached apparent equilibrium values of 6% and 23% respectively. The apparent equilibrium values of MIT, DIT and T_4 were similar to those found in rats fed a diet low in iodine but the T_3 value was slightly higher.

Absolute specific activity of the iodoamino acids. ($\mu c.^{131}I/\mu g.^{127}I$)

The specific activity-time curves of the various thyroidal iodoamino acids, fractionated from the enzymic hydrolysates by paper chromatography, are illustrated in Fig.28 and 29. The data used in plotting these curves is

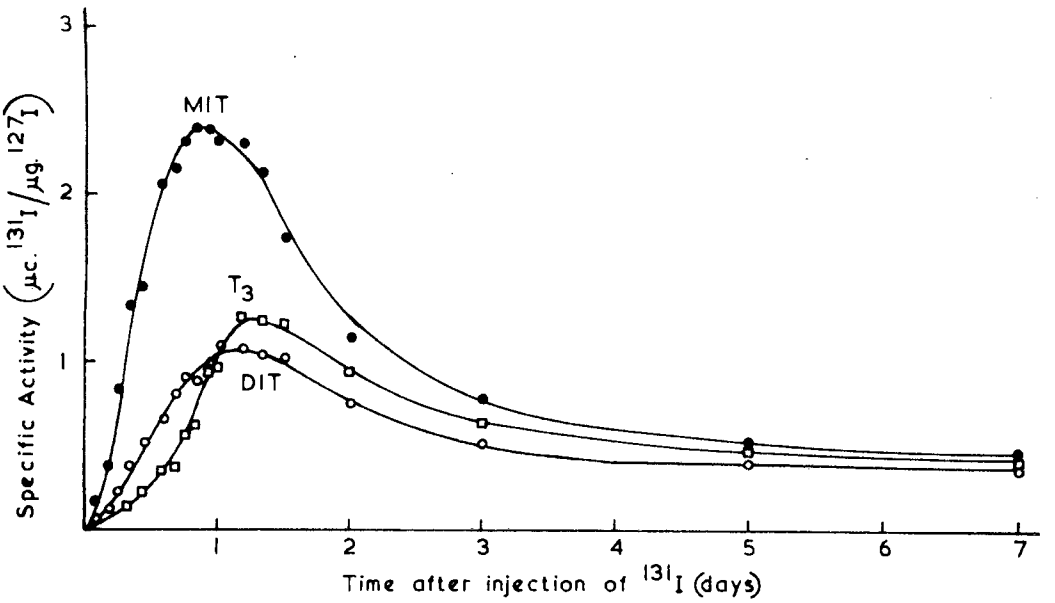

Fig. 28. Absolute specific activities of the iodine in MIT, DIT and T_3 in thyroid hydrolysates of rats injected with 0.5 mg. PTU 5 min. after administration of ^{131}I. Rats killed at intervals 2 - 168 hr. after injection of ^{131}I.

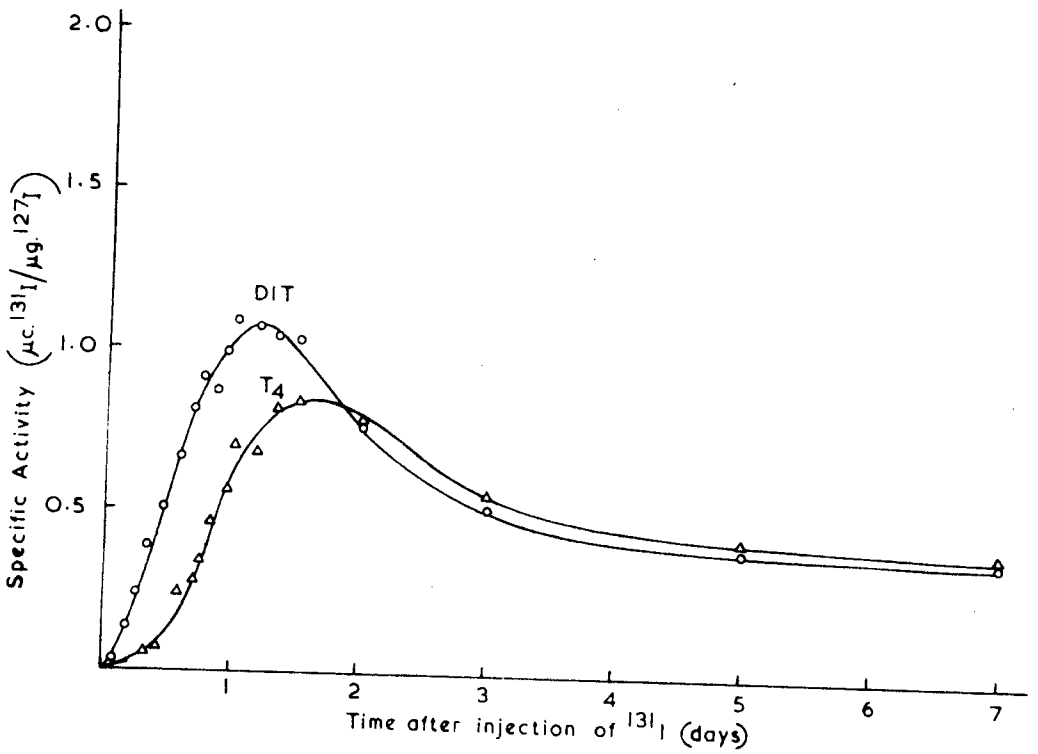

Fig. 29. Absolute specific activities of the iodine in DIT and T_4 in the hydrolysates of rats injected with 0.5 mg. PTU 5 min. after injection of ^{131}I. Rats killed at intervals 2 - 168 hr. after injection of ^{131}I.

presented in TABLE 25.

Fig.28. shows that the specific activity of MIT greatly exceeded that of DIT for the period up to approximately 36 hr. Thereafter the specific activity of MIT and DIT gradually became similar and at 168 hr. the S ratio was 1.17. At the earlier intervals the S ratio was as high as 2.5 - 3.5. The peak specific activity of MIT occurred at 20 - 22 hr. whereas the maximum specific activity of DIT was reached much later at 24 - 32 hr., when the specific activity of MIT was already decreasing rapidly.

The specific activity of T_3 exceeded that of T_4 during the entire period of the experiment. However, at the earlier intervals the T_3/T_4 ratio was much higher. The peak specific activities of T_3 and T_4 occurred at approximately 36 hr. and 42 hr. respectively.

The rates of increase in the initial specific activity of the various iodoamino acids indicated that PTU inhibits the synthesis of the iodothyronines to a greater extent than it inhibits the synthesis of the iodotyrosines. The synthesis of T_3 and T_4 were both inhibited to a similar extent. The synthesis of MIT was effected very little whereas the synthesis of DIT was greatly reduced but to a lesser extent than the synthesis of the iodotyronines.

TABLE 25.

Absolute specific activity of iodine in the components of thyroid hydrolysates of rats injected with 0.5 mg. PTU 5 min. after administration of ^{131}I. The rats were killed at intervals 2 - 168 hr. after the injection of ^{131}I.

Interval after ^{131}I hr.	Specific activity ($\mu c.^{131}I/\mu g.^{127}I$) *				MIT/DIT	T_3/T_4
	MIT	DIT	T4	T3		
2	0.080	0.031	-	-	2.58	-
4	0.383	0.131	-	-	2.92	-
6	0.839	0.238	-	-	3.52	-
8	1.344	0.384	0.052	0.144	3.50	2.77
10	1.450	0.502	0.068	0.230	2.88	3.38
14	2.064	0.678	0.243	0.377	3.05	1.53
16	2.152	0.814	0.282	0.366	2.64	1.30
18	2.299	0.906	0.346	0.570	2.54	1.65
20	2.403	0.873	0.458	0.635	2.76	1.39
22	2.380	0.997	0.560	0.944	2.38	1.69
24	2.319	1.090	0.698	0.975	2.13	1.40
28	2.302	1.071	0.680	1.265	2.14	1.86
32	2.130	1.040	0.825	1.246	2.05	1.51
36	1.751	1.029	0.842	1.233	1.70	1.47
48	1.148	0.760	0.778	0.937	1.51	1.21
72	0.787	0.509	0.550	0.626	1.51	1.14
120	0.544	0.394	0.416	0.462	1.38	1.11
168	0.430	0.368	0.371	0.389	1.17	1.02

* Each value represents the mean of 6 rats.

The specific activity-time curves of DIT and T_4 (Fig. 29.) showed a precursor-product relationship. At early intervals (2 - 36 hr.) the specific activity of DIT was greater than that of T_4 but after 48 hr. the specific activity of T_4 exceeded that of DIT. The DIT curve cut the T_4 curve almost at its maxium.

Similarly, after 24 hr. the specific activity-time curves of MIT, DIT and T_3 (Fig. 28.), indicated a relationship which would be expected if T_3 was synthesized by the coupling of MIT and DIT.

EXPERIMENT 2 : PTU administered before and after injection of ^{131}I.

Uptake of ^{131}I.

The uptake of ^{131}I by the thyroid gland reached a maxium of 3.20% of the injected dose at 12 hr. and fell to 0.34% after 168 hr. The release curve (Fig. 30.) was biphasic in nature, the biological half-life of the thyroidal ^{131}I being 1.17 days for the first exponential and 4.04 days for the second exponential.

Distribution of ^{131}I in the thyroid gland.

a) Unhydrolysed glands.

The distribution of ^{131}I in the thyroid homo-

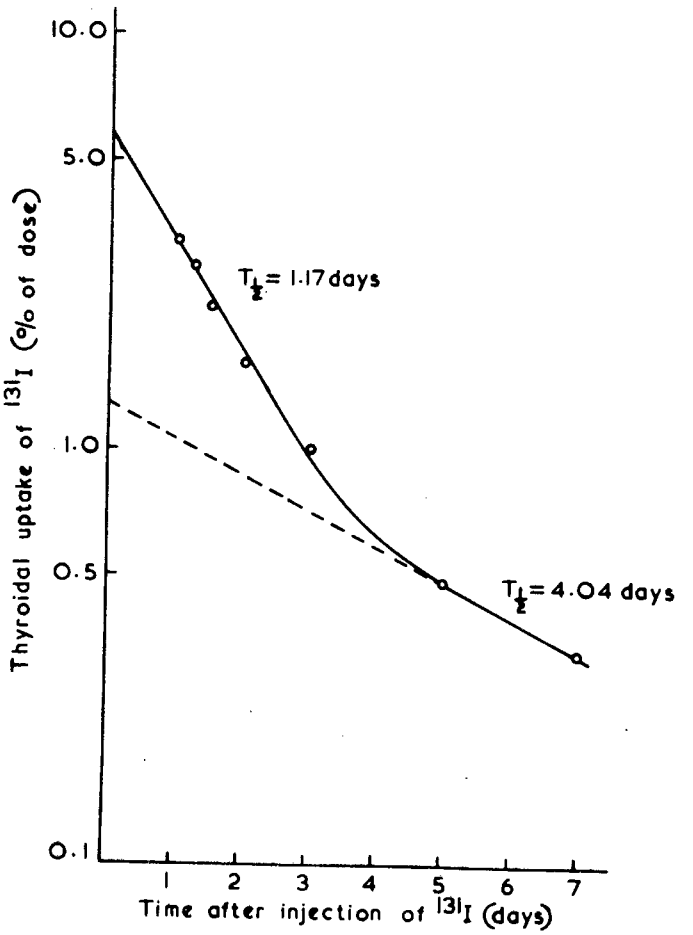

Fig. 30. The release of ^{131}I from the thyroid glands of rats administered PTU before and after the injection of ^{131}I (see dose schedule outlined in Methods, Chapter 5).

genates of rats killed 48 hr. and 72 hr. after injection was determined by electrophoresis. The inorganic ^{131}I iodide accounted for 1.25% and 1.38% of the total thyroidal ^{131}I at 48 hr. and 72 hr. relatively.

b) Hydrolysed glands.

The distribution of ^{131}I between the various iodoamino acids in the thyroid hydrolysates for the period 3 - 168 hr. after injection of ^{131}I is shown in Fig. 31 . The data used for constructing the curves is presented in TABLE 26.

The percentage of the total thyroidal ^{131}I present as iodotyrosines was found greatly to exceed that of the iodothyronines at all intervals. For the duration of the experiment, the percentage ^{131}I in MIT was greater than that of DIT and also labelled T_4 exceeded that of T_3. The R value was much higher at the early intervals whereas the T_3/T_4 ratio remained relatively constant (TABLE 26). Although the percentage ^{131}I present in the iodothyronine fraction was greatly decreased, the ratio of labelled T_3 to T_4 was increased. MIT became maximally labelled at approximately 24 hr. and then diminished slightly with time. The relative abundances of DIT, T_3 and T_4 became maximal at 48 hr. The equilibrium percentage distribution of ^{131}I in MIT, DIT, T_4 and T_3 was 43.6, 30.8, 7.1 and 2.5 respectively.

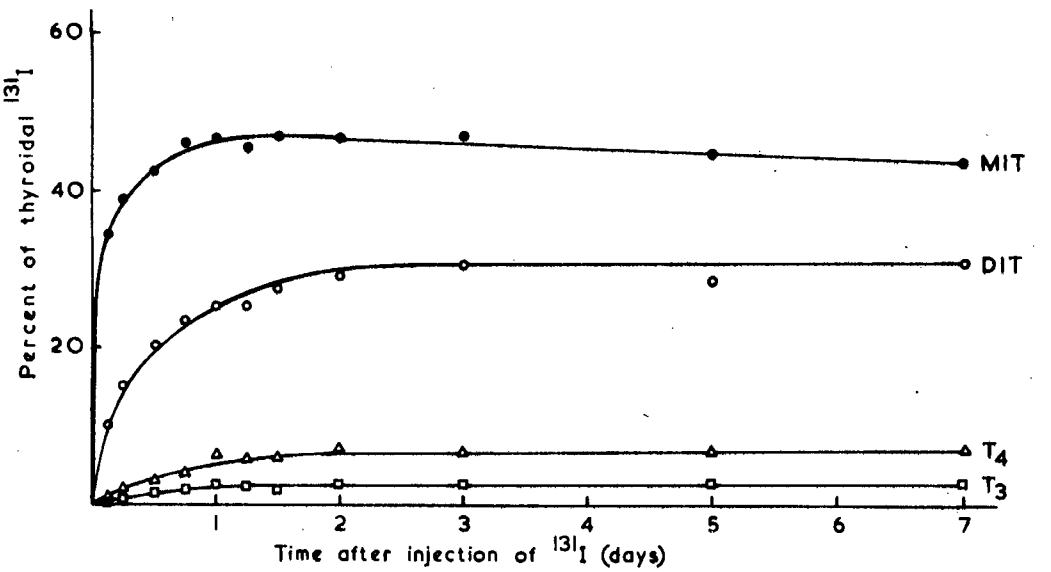

Fig. 31. Distribution of 131I between the various iodoamino acids in the hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). Rats killed at intervals 3 - 168 hr. after injection of ^{131}I.

TABLE 26.

The distribution of ^{131}I between the iodoamino acids of thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). The rats were killed at intervals 3 - 168 hr. after injection of ^{131}I.

Interval after ^{131}I hr.	% distribution of ^{131}I*					MIT/DIT	T_3/T_4
	I	MIT	DIT	T_4	T_3		
3	48.32	34.54	10.07	0.71	-	3.42	-
6	36.90	38.97	15.25	1.55	0.44	2.55	0.28
12	26.07	42.50	20.22	2.60	1.66	2.10	0.64
18	17.43	46.12	23.75	3.89	0.99	1.95	0.25
24	12.58	46.40	25.41	6.31	2.41	1.82	0.38
30	13.83	45.71	25.59	5.94	2.29	1.79	0.39
36	10.64	46.90	27.92	5.92	1.86	1.68	0.31
48	8.56	46.77	29.50	7.05	2.71	1.59	0.38
72	7.42	46.53	30.78	6.46	2.43	1.51	0.38
120	8.08	44.60	28.58	6.84	2.67	1.57	0.39
168	8.29	43.62	30.79	7.09	2.51	1.42	0.35

* Each value represents the mean of 6 rats.

The inorganic ^{131}I resolved from the thyroid hydrolysates by paper chromatography was found to decrease in an exponential manner at the early intervals and then reached an apparent equilibrium value of 8% at 48 hr. (TABLE 26).

The total absolute ratioactivity in each of the MIT, DIT, T_4 and T_3 pools was measured during the period 24 - 168 hr. Fig. 32. shows that the rate of loss of ^{131}I from each of the iodoamino acid pools is the same and that the loss of ^{131}I from each pool is biphasic in nature. From the slopes of the curves illustrated in Fig. 32, the mean rate of loss of ^{131}I from the iodoamino acid pools during the interval 24 - 48 hr. was 0.693/day whereas during the period 72 - 168 hr. the mean loss of ^{131}I was 0.275/day.

Distribution of ^{127}I in the thyroid hydrolysates.

The quantitative distribution of ^{127}I between the various iodoamino acids in the thyroid hydrolysates of rats killed during the interval 24 - 168 hr. after injection of ^{131}I is shown in TABLE 27. The ^{127}I content of each of the iodoamino acid components was found to decrease steadily at the same rate (Fig. 33). The mean rate of loss of ^{127}I from the iodoamino acid pools measured from the slopes of the curves illustrated in

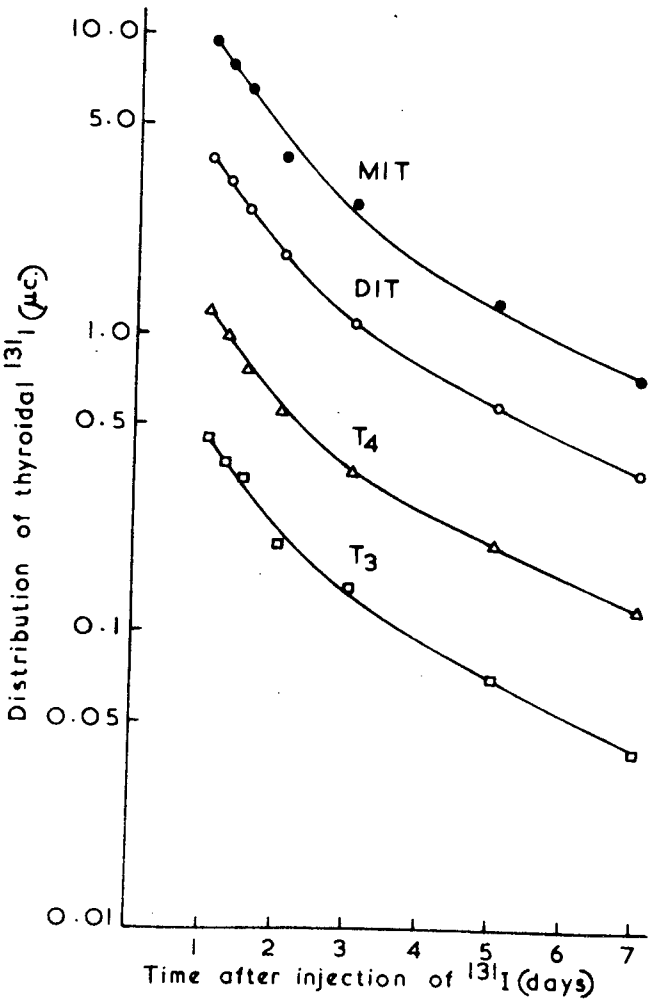

Fig. 32. Variation in total absolute radioactivity (μc.) of MIT, DIT, T_4 and T_3 in the thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). Rats killed at intervals 24 - 168 hr. after injection of ^{131}I.

TABLE 27.

The quantitative distribution of stable iodine between the iodoamino acids of thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I(see dose schedule outline under Methods, Chapter 5). The rats were killed at intervals 24 - 168 hr. after injection of ^{131}I.

Interval after ^{131}I hr.	Distribution of ^{127}I (µg.)*				Total ^{127}I µg.
	MIT	DIT	T_4	T_3	
24	2.263	3.905	1.119	0.226	7.513
30	2.131	3.838	0.942	0.200	7.111
36	2.042	3.589	0.943	0.209	6.783
48	1.820	3.387	0.814	0.171	6.192
72	1.693	3.016	0.722	0.167	5.598
120	1.187	1.951	0.546	0.112	3.796
168	1.006	1.598	0.372	0.092	3.068

* Each value represents the mean of 6 rats.

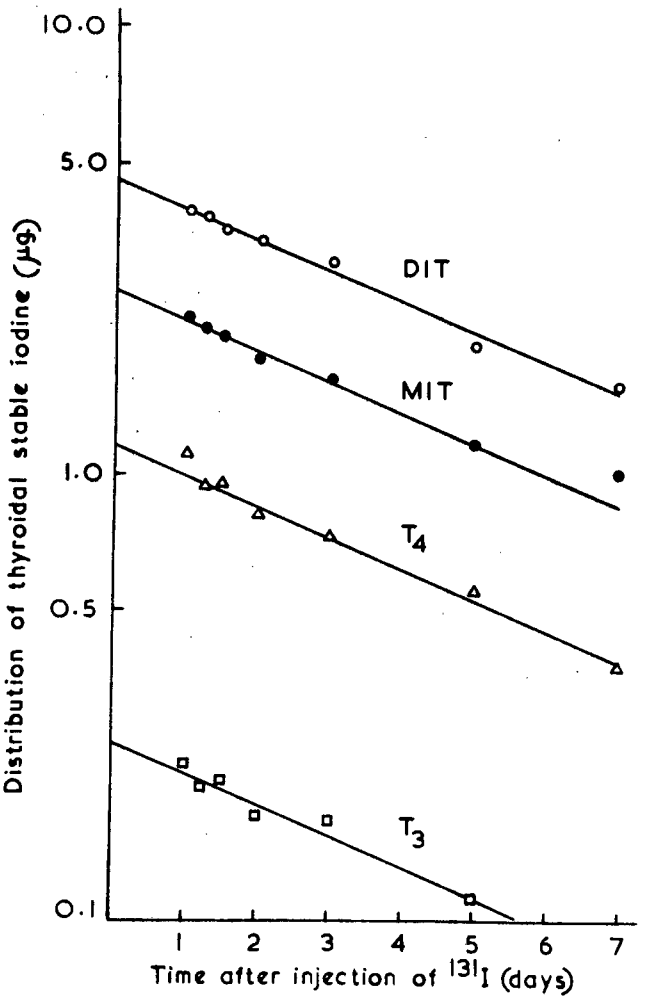

Fig. 33. Quantitative distribution of ^{127}I between the various iodoamino acids in the hydrolysates of rats administered PTU before and after injection of ^{131}I. (see dose schedule outlined under Methods, Chapter 5). Rats killed at intervals 24 - 168 hr. after injection of ^{131}I.

Fig. 33. was 0.147/day. Although the ^{127}I content of the iodoamino acids was found to decrease with time, the relative distributions of ^{127}I between the different iodoamino acids remain constant (TABLE 28).

Comparison of the mean relative distributions of the ^{127}I between the four iodoamino acids with that found in untreated rats (Chapter 3, Part II) indicated that the mean percentages of ^{127}I in MIT, DIT and T_3 were similar to those of untreated rats. However, the mean percentage of ^{127}I in T_4 was considerably lower, the values for PTU-treated and normal rats being 13.5% and 22.5% respectively.

Absolute specific activity of the iodocompounds. ($\mu c.^{131}I/\mu g.^{127}I$)

The specific activities of the inorganic iodide of unhydrolysed thyroid homogenates of rats killed 3 - 168 hr. after injection of ^{131}I were determined using electrophoresis (TABLE 29).

The specific activity curve of the iodide was found to cut the specific activity curve of MIT at approximately its maxium but did not cut the specific-activity curve of DIT (Fig. 34). During the interval 36 - 168 hr., the specific activity of the iodide fraction attained values which would be expected if the iodide arose by deiodination of MIT and DIT and if they were deiodinated

TABLE 28.

The relative distribution of stable iodine between the iodoamino acids of thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). The rats were killed at intervals 24 - 168 hr. after injection of ^{131}I.

Interval after ^{131}I hr.	Distribution of ^{127}I (% of total ^{127}I) *			
	MIT	DIT	T4	T3
24	30.1	51.9	14.9	3.1
30	30.0	54.0	13.2	2.8
36	30.1	53.0	13.9	3.1
48	29.4	54.8	13.2	2.6
72	30.3	53.8	12.9	3.0
120	31.3	51.4	14.4	2.9
168	32.9	52.0	12.1	3.0

* Each value represents the mean of 6 rats.

TABLE 29.

Absolute specific activity of iodine in the components of thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). The rats were killed at intervals 3 - 168 hr. after injection of ^{131}I.

Interval after ^{131}I hr.	Specific activity ($\mu c.^{131}I/\mu g.^{127}I$) *					MIT/DIT	T_3/T_4
	I (electrophoresis)	MIT	DIT	T4	T3		
3	6.943	0.910	0.208	0.081	0.168	4.34	2.13
6	5.871	2.019	0.594	0.179	0.332	3.42	1.84
12	4.929	3.452	0.881	0.480	0.917	3.92	1.92
18	3.402	4.344	1.205	0.953	1.701	3.60	1.79
24	2.456	4.228	1.320	1.160	2.135	3.21	1.84
30	2.173	3.749	1.170	1.141	2.03	3.21	1.78
36	1.939	3.375	0.992	0.905	1.833	3.38	2.03
48	1.116	2.191	0.754	0.753	1.249	2.88	1.68
72	0.799	1.669	0.543	0.541	0.865	3.07	1.59
120	0.571	1.093	0.403	0.409	0.616	2.75	1.49
168	0.447	0.857	0.302	0.306	0.484	2.87	1.55

* Each value in the table represents the mean of 6 rats.

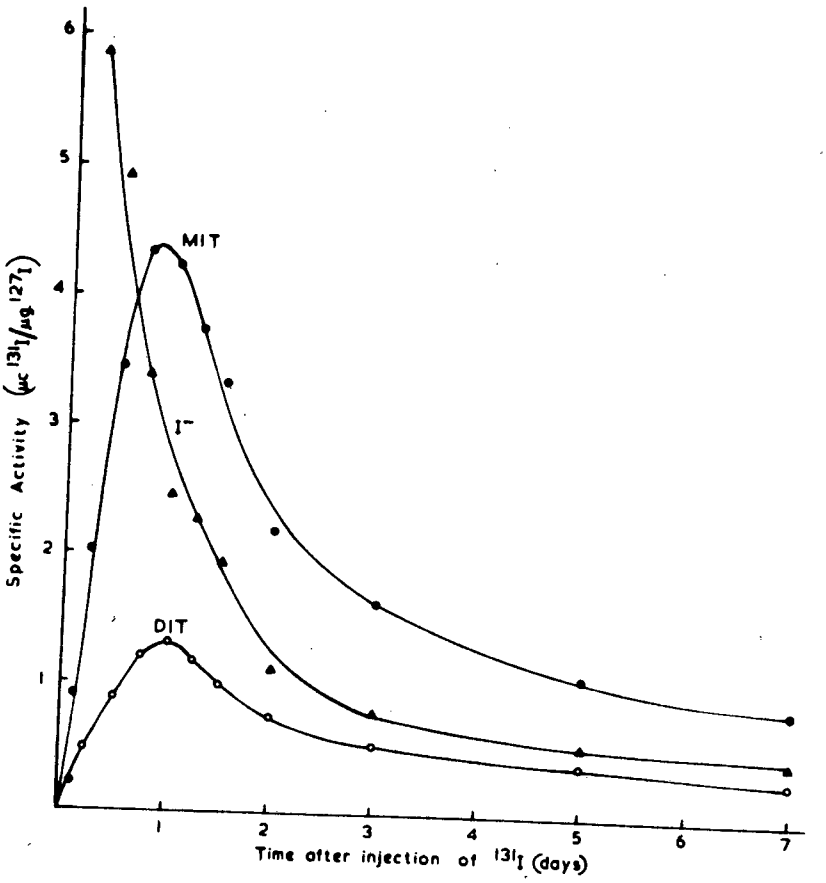

Fig. 34. Absolute specific activities of inorganic iodide and of the iodine in MIT and DIT in the thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). Rats killed at intervals 3 - 168 hr. after injection of ^{131}I.

at approximately the same rate.

The variations in specific activity of the iodine in the different thyroidal iodoamino acids were measured as a function of time (Fig. 35 and 36). The data used for the construction of the curves illustrated in Fig. 35 and 36. are presented in TABLE 29.

Fig. 35. shows that the specific activity of MIT greatly exceeded that of DIT for the entire duration of the experiment. Although the S ratio was higher at the early intervals, it became relatively constant after 48 hr. (TABLE 29). The maxium specific activity of MIT occurred at 18 - 21 hr. whereas the peak specific activity of DIT was much later, occurring at 24 hr.

The specific activity of T_3 was at all intervals much higher than both that of DIT and T_4, except for the short period 3 - 12 hr. when DIT exceeded that of T_3. Although at the earlier intervals the specific activity of T_4 was less than that of DIT, it increased virtually in parallel with DIT. At approximately 48 hr. the specific activity of T_4 became equal to that of DIT and remained equal throughout the experiment (Fig. 36). The peak specific activities of T_3 and T_4 occurred at the same time between 24 - 30 hr. The ratio of the specific activities of T_3 to T_4 were greatly enhanced and at earlier times were slightly higher than at later

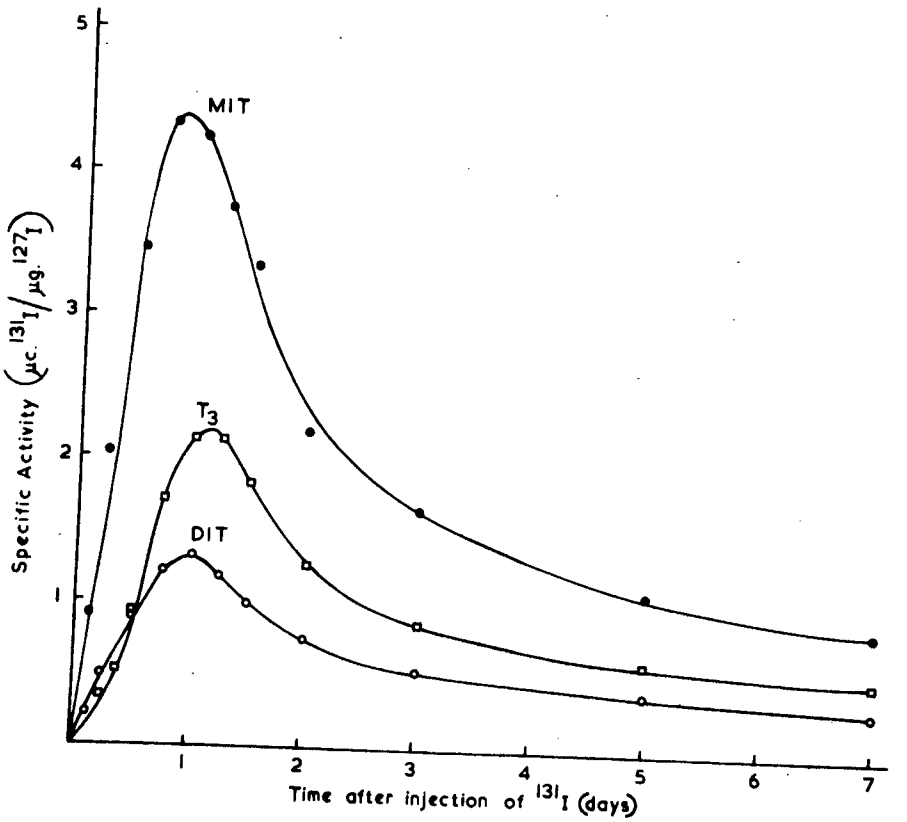

Fig. 35. Absolute specific activities of MIT, DIT and T_3 in the thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). Rats killed at intervals 3 - 168 hr. after injection of ^{131}I.

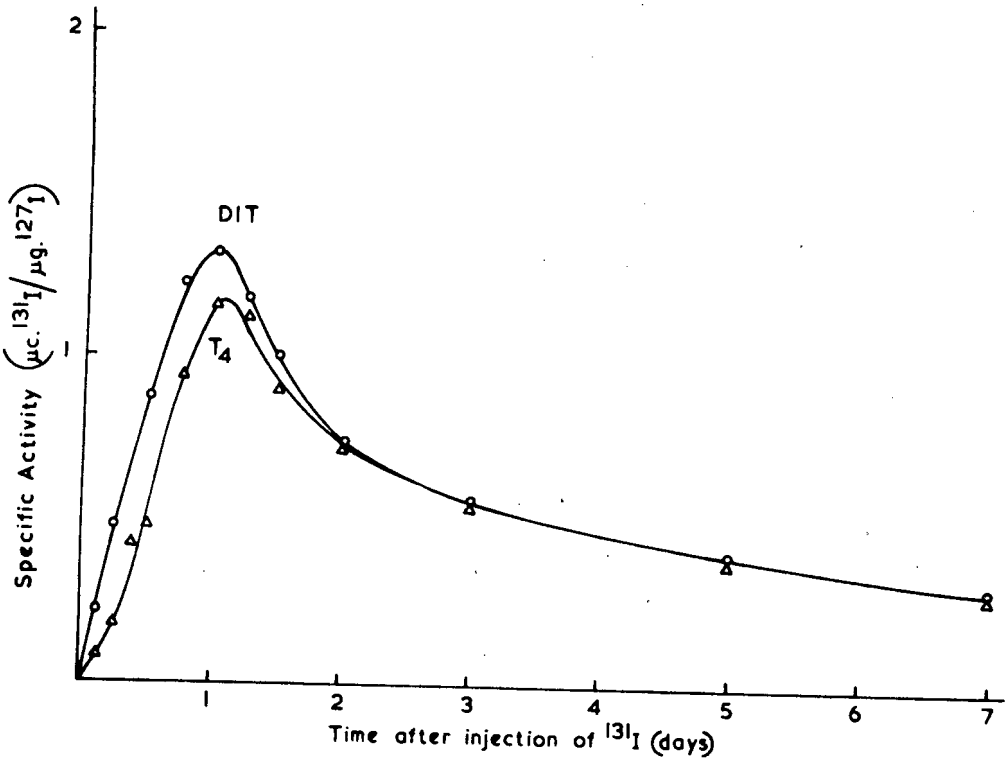

Fig. 36. Absolute specific activities of DIT and T_4 in the thyroid hydrolysates of rats administered PTU before and after injection of ^{131}I (see dose schedule outlined under Methods, Chapter 5). Rats killed at intervals 3 - 168 hr. after injection of ^{131}I.

intervals (TABLE 29).

From the initial increase in the specific activities of the individual iodoamino acids it is concluded that the formation of iodothyronines are inhibited to a greater extent than that of the iodotyrosines. The data further indicate that PTU affects thyroidal iodination of tyrosine in the rat to a lesser extent than that of MIT.

DISCUSSION.

Although the experiments of the present study on PTU-treated rats were primarly designed to produce a permanent situation in which the specific activity of MIT would be greatly different from DIT, it was also possible to show that the fractional rates of loss of ^{127}I and of ^{131}I from the thyroid glands of PTU-treated rats were different. It may be seen from the slopes of the curves, illustrating the variation in ^{131}I and ^{127}I content of the MIT, DIT, T_4 and T_3 pools as a function of time (Fig. 32 and Fig. 33.), that the rate of loss of ^{131}I from each of the iodoamino acid pools is much greater than the rate of loss of ^{127}I. In fact, the rate of loss of ^{131}I is approximately five times greater

than that of ^{127}I. These findings confirm the results of Schneider (1964) and Rosenberg, La Roche and Ehlert (1966) that thyroidal iodine is heterogeneous with respect to turnover. In addition, the present data indicate that the rate of loss of either the ^{131}I or the ^{127}I from each of the iodoamino acids pools is the same. Therefore, although within each individual pool the turnover of ^{131}I is heterogeneous with respect to the ^{127}I, the rates of turnover of the individual iodoamino acid pools with respect to each are the same.

The curves representing the loss of ^{131}I from the individual iodoamino acid fractions were biphasic in nature. This could arise from the partial blocking of the gland so that at later intervals, when the ^{131}I content of the gland is low, the reutilization of the ^{131}I secreted from the gland as hormone may become significant and cause stabilization of the loss of ^{131}I at a slower rate. This possibility is not favoured since comparison of the uptakes of ^{131}I of untreated and PTU-treated rats reveal that the degree of blocking is over 80% and also since the uptake of ^{131}I was still approximately 40% of the maxium when stabilization of the loss of ^{131}I first became significant in the PTU-treated rats. However, histological sections of the glands from PTU-treated rats indicated that the ratio of small to large follicles

is greatly increased. In the light of the findings of Nadler, Leblond and Bogoroch (1954) this could result in a more rapid uptake and release of ^{131}I from the small follicles at the earlier intervals after injection of ^{131}I, but as the ^{131}I is lost from the small follicles, the rate of loss of ^{131}I from the various iodoamino acid pools will decrease and tend towards that of the large follicles which is probably similar to the rate of loss of ^{127}I. Consequently, since the ^{127}I content of the small follicles is much less than that of the large follicles and since their turnover rate is much faster, the iodine which is lost at early intervals will have a higher specific activity than that released later. It is therefore possible that the PTU by increasing the proportion of small to large follicles may accentuate or even induce the hetereogeneity found. The latter possibility is unlikely since the preferential release of newly labelled iodine can result simply from the slow diffusion of the thyroglobulin molecules away from the site of iodination at the cell-colloid interface.

It was not possible in rats on an adequate or low iodine diet to obtain a situation in which the specific activities of MIT and DIT were considerably different. However, treatment with PTU caused significant changes in the specific activities of MIT and DIT,

the specific activity of MIT greatly exceeding that of DIT. After a single dose of PTU the changes were evident for 3 - 5 days but could be maintained for as long as the administration of PTU was continued (Experiment 2).

The specific activity curves of the thyroidal iodide and of MIT showed that expected simple precursor-product relationship but the iodide curve did not cut the specific activity curve of DIT at its maxium. Therefore it is reasonable to suppose that tyrosine is not directly labelled to form DIT and that MIT is a stable intermediate in the biosynthesis of DIT.

Although the variation in the relative abundances in MIT and DIT, showed a simple precursor-product relationship (Fig. 27), it cannot be considered to be a genuine precursor-relationship since the pool sizes of MIT and DIT were probably changing at the time. The apparent precursor-product relationship may also have arisen due to the partial inhibition of the iodination of MIT by PTU. This could cause the accumulation of MIT but as the effect of the single dose of PTU wore off, the MIT would again be normally converted to DIT. The net effect would be that at early intervals a large amount of labelled MIT and a small amount of labelled DIT would be present. However, with increasing time the percentage labelled MIT would decrease while the per-

centage labelled DIT would increase toward their normal equilibrium distributions. It is therefore likely that the MIT and DIT curves illustrated in Fig. 27. show indirectly that MIT is the precursor of DIT.

The S ratio was calculated from the equation A (Chapter 3 , Part II, Discussion). Using the value of 1.73 for the ratio $\frac{y_1}{y_2}$ (obtained from the rates of increase in the total activity of the MIT and DIT pools) and values of 2.26 µg. and 3.90 µg. for the total stable iodine in the MIT and DIT pools 24 hr. after ^{131}I, the S ratio was found to be 2.98. The calculated S ratio is in close agreement with the value found experimentally at 24 hr. (3.21). Since the MIT/DIT ratio of the stable iodine remained relatively constant, the calculated S value would be approximately 3 for intervals from 24 hr. to 168 hr. This agrees closely with the values obtained experimentally. The experimental S values probably remained constant because the intrathyroidal recycling of iodine was almost completely prevented by PTU.

The findings of the present experiments on PTU-treated rats show that, even with gross differences in the specific activities of MIT and DIT, the kinetics of labelling of DIT and T_4 were consistent with DIT being the biological precursor of T_4. The data of Experiment 2 indicate that it is highly unlikely that

T_4 arises from the iodination of T_3 since the specific activity of T_3 was at all times considerably greater than that of T_4 (at least 1.5 times greater). Since the specific activity of iodide was similar to that of T_3 for the period 30 - 168 hr., it would be expected that during this interval, the specific activity of T_4 would be similar to T_3 if T_4 was synthesized by iodination of T_3. Even if unlabelled iodide was incorporated into T_3 the specific activity of T_4 would be considerably higher than that found experimentally.

The kinetics of labelling of T_3 was compatible with the view that T_3 was formed by the coupling of MIT and DIT. If T_3 was synthesized via coupling, it would be expected that the specific activity curve of T_3 would lie between the curves of MIT and DIT but closer to the DIT curve. This was in fact found. The results of Experiment 2 eliminate the possibility of T_3 arising from T_4 by deiodination since at all times the specific activity of T_3 was considerably higher than that of T_4.

Since the experimental conditions were such as to produce a situation in which the specific activities of MIT and DIT were grossly different, it was possible at the intervals when the equilibrium distribution of ^{131}I was attained, to compare the calculated specific activities of T_3 and T_4 for each of the proposed biosyn-

thetic pathways with those obtained experimentally (Chapter 3 , Part II, Discussion, Biosynthesis of Iodothyronines).

TABLES 30 and 31. show the specific activity data of inorganic iodide and of the various iodoamino acids found in the thyroid glands of individual rats killed in Experiment 1 (36 - 168 hr.) and Experiment 2 (24 - 168 hr.). The specific activities of T_3 and of T_4 were calculated from the specific activities of the suspected precursors for each of the pathways proposed for T_3 and T_4 synthesis.

If T_3 is synthesized by the coupling of MIT and DIT (Pathway A1), one would expect its specific activity to be $\frac{m + 2d}{3}$ (where m and d are the specific activities of MIT and DIT). However, if T_3 arose by the deiodination of T_4 (Pathway A2) the specific activity of T_3 would be the same as that of T_4. Similarly, if T_4 was formed by the coupling of two DIT molecules (Pathway B1) it would be expected that the specific activity of T_4 would be equal to that of DIT. On the other hand if T_3 was iodinated to form T_4 (Pathway B2), the specific activity of T_4 would be $\frac{3x + y}{4}$ (where x is the specific activity of the iodine being incorporated into T_3).

A student's t analysis was carried out to test the differences between the experimental specific

TABLE 31 (cont).

Time	MEASURED SPECIFIC ACTIVITY ($\mu c.^{131}I/\mu g.^{127}I$)					CALCULATED T_3 SPECIFIC ACTIVITY		CALCULATED T_4 SPECIFIC ACTIVITY	
	I	MIT	DIT	T_4	T_3	Pathway A1 (MIT + DIT $\rightarrow T_3$)	Pathway A2 ($T_4 \rightarrow T_3$ + I)	Pathway B1 (DIT + DIT $\rightarrow T_4$)	Pathway B2 (T_3 + I $\rightarrow T_4$)
72 hr.	0.804	1.808	0.571	0.579	0.943	0.950	0.579	0.571	0.908
	0.859	1.665	0.528	0.522	0.908	0.907	0.522	0.528	0.896
	0.737	1.561	0.484	0.471	0.821	0.843	0.471	0.484	0.800
	0.835	1.582	0.550	0.567	0.866	0.894	0.567	0.550	0.858
	0.773	0.710	0.537	0.543	0.772	0.928	0.543	0.537	0.772
	0.786	1.692	0.588	0.576	0.880	0.956	0.576	0.588	0.856
120	0.524	1.229	0.313	0.314	0.621	0.665	0.314	0.313	0.594
	0.566	0.986	0.402	0.401	0.605	0.596	0.401	0.402	0.595
	0.643	1.001	0.335	0.357	0.549	0.557	0.357	0.335	0.575
	0.464	1.215	0.441	0.472	0.670	0.699	0.472	0.441	0.618
	0.719	1.144	0.424	0.436	0.637	0.664	0.436	0.424	0.657
	0.510	0.935	0.458	0.474	0.614	0.617	0.474	0.458	0.588
168	0.545	0.881	0.239	0.259	0.459	0.453	0.259	0.239	0.480
	0.412	0.778	0.316	0.328	0.486	0.470	0.328	0.316	0.468
	0.363	0.874	0.273	0.281	0.431	0.474	0.281	0.274	0.414
	0.358	0.857	0.320	0.341	0.484	0.499	0.341	0.320	0.452
	0.478	0.833	0.365	0.335	0.537	0.521	0.335	0.365	0.522
	0.526	0.919	0.298	0.292	0.517	0.505	0.292	0.298	0.519
MEAN				0.748	1.316	1.339	0.772	0.758	1.326
STANDARD DEVIATION				0.333	0.651	0.668	0.378	0.376	0.682

TABLE 30.

Comparison of the measured specific activities of T_3 and T_4 with those calculated for the different theories of thyroid hormone biosynthesis. Rats were injected with 0.5 mg. PTU 5 min. after administration of ^{131}I and killed at intervals 36 - 168 hr. after ^{131}I.

Interval after ^{131}I hr.	MEASURED SPECIFIC ACTIVITY ($\mu c.^{131}I/\mu g.^{127}I$)					CALCULATED T_3 SPECIFIC ACTIVITY		CALCULATED T_4 SPECIFIC ACTIVITY	
	I	MIT	DIT	T_4	T_3	Pathway A1 (MIT + DIT $\longrightarrow T_3$)	Pathway A2 ($T_4 \rightarrow T_3$ + I)	Pathway B1 (DIT + DIT $\longrightarrow T_4$)	Pathway B2 (T_3 + I $\rightarrow T_4$)
36	-	1.420	1.227	0.873	1.267	1.291	0.873	-	-
	-	1.665	1.079	0.849	1.241	1.274	0.849	-	-
	-	1.884	0.923	0.852	1.163	1.243	0.852	-	-
	-	1.730	1.012	0.791	1.211	1.251	0.791	-	-
	-	1.827	1.037	0.860	1.294	1.301	0.860	-	-
	-	1.980	0.896	0.827	1.223	1.257	0.827	-	-
48	0.872	1.025	0.742	0.698	0.921	0.836	0.698	0.742	0.909
	0.854	1.929	0.790	0.759	0.895	0.836	0.759	0.790	0.885
	0.830	1.413	0.674	0.826	0.942	0.920	0.826	0.674	0.916
	0.916	1.264	0.808	0.779	0.988	0.960	0.779	0.808	0.970
	0.793	1.059	0.777	0.807	0.931	0.871	0.807	0.777	0.896
	0.889	1.198	0.769	0.799	0.945	0.912	0.799	0.769	0.931
72	0.729	0.810	0.518	0.566	0.632	0.615	0.566	0.518	0.656
	0.698	0.717	0.507	0.549	0.590	0.577	0.549	0.507	0.617
	0.731	0.695	0.590	0.505	0.655	0.625	0.505	0.590	0.674
	0.608	0.784	0.473	0.497	0.592	0.577	0.497	0.473	0.596
	0.643	0.855	0.444	0.574	0.627	0.581	0.574	0.444	0.641
	0.629	0.861	0.522	0.609	0.660	0.635	0.609	0.522	0.652
120	0.407	0.528	0.459	0.383	0.446	0.449	0.383	0.459	0.451
	0.474	0.506	0.402	0.419	0.471	0.437	0.419	0.402	0.472
	0.433	0.547	0.424	0.459	0.473	0.465	0.459	0.424	0.463
	0.489	0.587	0.351	0.362	0.444	0.430	0.362	0.351	0.455
	0.443	0.495	0.382	0.436	0.460	0.420	0.436	0.382	0.456
	0.436	0.599	0.346	0.437	0.458	0.430	0.437	0.346	0.452

TABLE 30 (cont.)

Interval after ^{131}I hr.	MEASURED SPECIFIC ACTIVITY ($\mu c.^{131}I/\mu g.^{127}I$)					CALCULATED T_3 SPECIFIC ACTIVITY		CALCULATED T_4 SPECIFIC ACTIVITY	
	I	MIT	DIT	T_4	T_3	Pathway A1 (MIT + DIT $\rightarrow T_3$)	Pathway A2 ($T_4 \rightarrow T_3$ + I)	Pathway B1 (DIT + DIT $\rightarrow T_4$)	Pathway B2 (T_3 + I $\rightarrow T_4$)
168	0.413	0.454	0.305	0.333	0.351	0.355	0.333	0.305	0.367
	0.460	0.407	0.379	0.365	0.385	0.388	0.365	0.379	0.404
	0.386	0.385	0.410	0.393	0.408	0.402	0.393	0.410	0.402
	0.401	0.469	0.357	0.370	0.394	0.394	0.370	0.357	0.396
	0.455	0.437	0.389	0.399	0.420	0.405	0.399	0.389	0.429
	0.429	0.428	0.368	0.356	0.376	0.388	0.356	0.368	0.389
	MEAN			0.528	0.729	0.717	0.591	0.508	0.603
	STANDARD DEVIATION			0.166	0.322	0.333	0.196	0.164	0.208
				(48-168 hr.)					

TABLE 31.

Comparison of the measured specific activities of T_3 and T_4 with those calculated for the different theories of thyroid hormone biosynthesis. Rats administered PTU before and after injection of ^{131}I and killed at intervals 24 - 168 hr. after ^{131}I.

	MEASURED SPECIFIC ACTIVITY ($\mu c.^{131}I/\mu g.^{127}I$)					CALCULATED T_3 SPECIFIC ACTIVITY		CALCULATED T_4 SPECIFIC ACTIVITY	
Time	I	MIT	DIT	T_4	T_3	Pathway A1 (MIT + DIT → T_3)	Pathway A2 (T_4 → T_3 + I)	Pathway B1 (DIT + DIT → T_4)	Pathway B2 (T_3 + I → T_4)
24 hrs.	2.016	4.243	1.380	1.254	2.240	2.334	1.254	1.380	2.184
	1.893	4.255	1.345	1.342	2.154	2.315	1.342	1.345	2.089
	2.817	3.896	1.228	0.940	1.984	2.117	0.940	1.228	2.192
	2.430	4.476	1.402	1.350	2.342	2.427	1.350	1.402	2.364
	2.571	4.087	1.300	1.168	2.033	2.229	1.168	1.300	2.167
	3.009	4.415	1.265	1.044	2.057	2.315	1.044	1.265	2.295
30	2.115	2.859	1.182	1.164	1.728	1.741	1.164	1.182	1.825
	2.031	3.177	1.329	1.289	1.951	1.945	1.289	1.329	1.971
	2.567	4.235	1.076	1.070	2.135	2.129	1.070	1.076	2.243
	1.609	3.907	1.204	1.185	2.100	2.105	1.185	1.204	1.977
	1.940	3.961	1.123	1.125	2.072	2.069	1.125	1.123	2.039
	2.776	4.358	1.106	1.007	2.194	2.190	1.007	1.106	2.339
36	2.237	3.037	1.066	0.902	1.769	1.723	1.902	1.066	1.886
	1.514	3.623	0.950	0.875	1.875	1.841	0.875	0.950	1.785
	1.201	3.463	0.992	0.890	1.817	1.816	0.890	0.992	1.663
	2.094	3.620	0.893	0.880	1.892	1.802	0.880	0.893	1.942
	1.972	3.536	0.977	0.907	1.833	1.830	0.907	0.977	1.868
	2.616	2.973	1.062	0.941	1.794	1.699	0.941	1.062	1.999
48	1.731	2.375	0.701	0.708	1.267	1.259	0.708	0.701	1.383
	1.239	2.574	0.753	0.747	1.383	1.360	0.747	0.753	1.347
	0.824	2.219	0.725	0.720	1.239	1.223	0.720	0.725	1.135
	1.018	1.668	0.853	0.863	1.146	1.128	0.863	0.853	1.114
	0.743	1.819	0.670	0.664	1.080	1.053	0.664	0.670	0.996
	1.141	2.492	0.821	0.820	1.382	1.378	0.820	0.821	1.322

activities of T_3 and T_4 and specific activities calculated from the data of Experiment 1 and Experiment 2 for pathway A1, A2, B1 and B2. For example, using the data of Experiment 1 the difference between the measured T_3 value and that calculated for pathway A2 gave a t value of 5.33 which is highly significant, and so it was concluded that the biosynthesis of T_3 did not follow pathway A2. The difference between the measured T_3 value and the value calculated for pathway A1 was 1.85 which was not significant. Therefore the difference between the measured T_3 value and that calculated for pathway A1 was tested for correlation. The correlation was found to be 0.995 which is highly significant. It was therefore concluded that biosynthesis of T_3 followed pathway A1 (coupling of MIT and DIT).

Similarly, the results for Experiment 1 for T_4 showed that T_4 was synthesized via pathway B1 (coupling of DIT). Both conclusions drawn from Experiment 1 concerning the biosynthesis of T_3 and T_4 were confirmed by the results of Experiment 2. Details of the actual statistical data (computer analysis) are presented in TABLE 32.

TABLE 32.

t values and correlations of the experimental and calculated specific activities for the proposed pathways of the biosynthesis of T_3 and T_4. The data were obtained from rats administered either a single dose of PTU 5 min. after injection of ^{131}I (Experiment 1). or from rats administered PTU before and after injection of ^{131}I (Experiment 2).

Pathway A1 : MIT + DIT $\longrightarrow$ T_3

Pathway A2 : T_4 $\longrightarrow$ T_3 + I

Pathway B1 : DIT + DIT $\longrightarrow$ T_4

Pathway B2 : T_3 + I $\longrightarrow$ T_4

	t values		correlation	t values		correlation
	A1	A2	A1	B1	B2	B1
EXPERIMENT 1						
a) measured T_3	1.85	5.33	0.995	-	-	-
b) measured T_4	-	-	-	1.76	6.43	0.940
EXPERIMENT 2						
a) measured T_3	2.10	9.89	0.995	-	-	-
b) measured T_4	-	-	-	0.40	9.78	0.890

SUMMARY

1. Using a simple three-compartment model, equations have been obtained describing the rates of thyroidal uptake, secretion and reutilization of ^{131}I. The theoretical data were used to calculate the absolute rates of thyroidal trapping and the secretion of iodine, and the absolute rate of peripheral degradation of endogenously synthesized organically bound iodine in rats on a diet adequate in iodine.

2. An accurate and reliable alkaline ashing method for the quantitative measurement of stable iodine in the iodotyrosines and iodothyronines, separated from thyroid hydrolysates by paper chromatography, has been developed. This method was used in the determination of specific activities of ^{131}I-labelled iodoamino acids in the hydrolysates of thyroid glands of rats.

3. The kinetics of labelling of the different thyroidal iodoamino acids were investigated in rats on adequate and low-iodine diets and in propylthiouracil (PTU)-treated rats using (a) the distribution of ^{131}I between the iodoamino acids and (b) absolute specific activity measurements.

4. The relative distribution of ^{131}I between monoiodotyrosine (MIT) and di-iodotyrosine (DIT) was not consistent with a precursor-product relationship in rats on the adequate-iodine diet. The ratio of labelled MIT to labelled DIT (R values) decreased gradually from 0.83 at 30 sec. to 0.50 at 168 hr. In rats fed a low-iodine diet the percentage of labelled MIT exceeded that of DIT at early intervals whereas at later intervals the reverse was true. However, this finding was not regarded as being compatible with a precursor-product relationship since the specific activity of MIT exceeded that of DIT throughout the entire period of the experiment. The changing R values in rats injected with a single dose of PTU 5 min. after ^{131}I were found to be consistent with a simple precursor-product relationship between MIT and DIT. However, these findings were probably due to the effects of PTU.

Although no simple precursor-product relationship was found between MIT and DIT, the results provide evidence that MIT is a stable intermediate in the synthesis of DIT.

The iodothyronines represented a greater percentage of the total thyroidal ^{131}I in iodine deficient rats than in animals fed an adequate-iodine diet. The ratio of labelled tri-iodothyronine (T_3) to labelled

thyroxine (T_4) was increased in the low-iodine rats and since there was a corresponding increase in the R value the findings were interpreted as being compatible with the view that T_3 was synthesized by coupling of MIT and DIT. Rats maintained on PTU showed a similar increase in the $^{T3}/_{T4}$ ratio although the percentage of ^{131}I present in the iodothyronine was greatly decreased.

5. Estimations have been made of the specific activities of the iodine in the different iodoamino acids in the thyroid glands of rats on normal and low-iodine diets and in the glands of rats either administered a single dose of PTU or maintained on PTU.

Specific activity measurements were used to determine the pathways of T_3 or T_4 synthesis by two different methods: (a) By following the variation in the specific activity of the iodoamino acids as a function of time. From the data precursor-product relationship curves were constructed. (b) By comparing the specific activities of T_3 and T_4 calculated from the probable precursors for the different proposed pathways of synthesis with those found experimentally.

6. The specific activity data showed the following: a) No precursor-product relationship was found between MIT and DIT in rats fed a diet adequate in iodine. The

ratio of the specific activities of MIT to DIT (S values) decreased gradually from 1.85 at 30 sec. to approximately unity at 72 hr. and thereafter remained constant. A similar pattern of labelling was found in rats on a low-iodine diet. In order to explain the relatively constant S values, a scheme of reactions for the mechanism of iodination of MIT and DIT has been postulated.

In rats receiving PTU the S value was greatly increased. This increased S value could be maintained for as long as the PTU was administered.

b) DIT and T_4 showed a precursor-product relationship, even in PTU-treated rats in which the specific activities of MIT and DIT were greatly different. In rats on adequate and on low-iodine diets the specific activity of DIT exceeded that of T_4 at early intervals and then became equal to that of the T_4 between 72 - 120 hr.

c) In rats maintained on PTU the specific activity of T_3 was greater than that of T_4 for the entire duration of the experiment. These findings were regarded as being incompatible with the view that T_3 was formed by the deiodination of T_4. In PTU-treated rats the specific activity of T_3 varied at the same rate as MIT and DIT and in accordance with a relationship which would be

expected if T_3 was synthesized by the coupling of MIT and DIT.

d) A comparison of the calculated specific activities of T_3 or T_4 with those found experimentally could not be made in animals on adequate or low-iodine diets since the specific activities of all the iodoamino acids became equal. However, in PTU-treated rats these comparisons could be made since the S value was greatly increased. Only the specific activity of T_3 calculated for the coupling of MIT and DIT agreed closely with the specific activities of T_3 found experimentally. Similarly, only the specific activity of T_4 calculated for the coupling of two DIT molecules showed a close correlation with the experimental T_4 values.

THE RESULTS REPORTED IN THIS THESIS PROVIDE EVIDENCE,

A) THAT MIT IS THE PRECURSOR OF DIT.

B) THAT T_3 IS SYNTHESIZED FROM THE COUPLING OF ONE MOLECULE OF MIT AND ONE MOLECULE OF DIT.

C) THAT T_4 IS SYNTHESIZED FROM THE COUPLING OF TWO MOLECULES OF DIT.

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